Technische Universität Braunschweig

The impact of drought stress on monoterpene biosynthesis in sage (*Salvia officinalis*): Dehydrins and monoterpene synthases as molecular markers

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Alzahraa Radwan

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Dedicated to:

My family

Abstract:

The impact of drought stress on monoterpene biosynthesis in sage (*Salvia officinalis*):

Dehydrins and monoterpene synthases as molecular markers

The product quality of medicinal plants is determined by the quality and quantity of the particular natural products. Deliberate modifications require a comprehensive knowledge on the biosynthetic pathways and their regulations. In this context, the complex interactions between stress and secondary metabolism are of special interest. This study was aimed to elucidate exemplarily the impact of drought stress on the biosynthesis and accumulation of monoterpenes in sage (*Salvia officinalis*). For this, the applicability of dehydrins as molecular stress markers as well as the gene expression of monoterpene synthases had been studied.

Dehydrin gene SoDHN was isolated from sage leaves. The cDNA sequence exhibits a total length of 1000 bp with a putative open reading frame of 735 bp (accession number: AEB77936.1). SoDHN is constitutively expressed in leaves; however, its expression is significantly increased by drought stress. Western blot analysis revealed that the SoDHN protein also is already present in non-stressed leaves; nevertheless, the accumulation of dehydrin protein is significantly enhanced under drought stress. In contrast to the transient transcription, the abundance of the dehydrin protein remained stable throughout the entire period. Thus, for evaluating the stress status, in addition to the gene expression also the abundance of the protein has to be determined.

Drought stress also impacts on the expression of monoterpene synthases; e.g. the amounts of mRNA for bornyl diphosphate synthase and cineole synthase already are strongly enhanced 2h after detaching the leaves and reach a maximum after 6h. Obviously, the monoterpene biosynthesis is – apart from the "passive" enhancement due to the drought-related over-reduced states – also "actively" increased by enhancing the biosynthetic capacity. This points out that monoterpenes – apart from their ecological functions – also are relevant to the dissipation of the massive over-supply of energy generated in leaves under drought stress.

Zusammenfassung:

Der Einfluss von Trockenstress auf die Monoterpen-Biosynthese in Salbei (*Salvia officinalis*):

Dehydrine und Monoterpen-Synthasen als molekulare Marker

Die Produktqualität von Arzneipflanzen hängt von der Qualität und Quantität der jeweiligen Naturstoffe ab. Eine gezielte Beeinflussung setzt ein umfassendes Wissen über die Biosynthese und deren Regulation voraus. Dabei kommt den komplexen Wechselwirkungen zwischen Stress- und Sekundärstoffwechsel eine besondere Bedeutung zu. Diese Untersuchung zielte darauf ab, exemplarisch die Auswirkungen von Trockenstress auf die Biosynthese und Akkumulation von Monoterpenen in Salbei (*Salvia officinalis*) zu erfassen. Dabei wurde zum einen die Eignung der Dehydrine als molekulare Stressmarker untersucht und zum anderen die Expression der Monoterpen-Synthasen bestimmt.

Das Dehydrin-Gen SoDHN wurde aus Salbei-Blättern isoliert. Die cDNA-Sequenz mit einer Gesamtlänge von 1000 bp weist ein putatives offenes Leseraster von 735 bp auf (Zugangsnummer AEB77936.1). SoDHN ist in Blättern konstitutiv exprimiert, doch seine Expression wird bei Stress signifikant erhöht. Western-Blot-Analysen ergaben, dass auch die Menge des SoDHN-Proteins, das bereits in nicht gestressten Blättern vorhanden ist, bei Trockenstress deutlich gesteigert wird. Im Gegensatz zur transienten Genexpression, bleibt die Menge des Dehydrin-Proteins während des gesamten Zeitraums stabil. Zur Evaluierung des Stress-Status muss also neben der Genexpression auch die Abundanz des Proteins erfasst werden.

Trockenstress beeinflusst auch die Expression der Monoterpen-Synthasen; so werden z.B. die mRNA-Menge der Bornyldiphosphat-Synthase und der Cineol-Synthase bereits 2h nach dem Abtrennen der Blätter stark erhöht und erreichen ihr maximales Level nach 6h. Offensichtlich wird die Monoterpen-Biosynthese – neben der “passiven“ Steigerung aufgrund der Trockenstress-induzierten überreduzierten Zustände – auch "aktiv" durch die Erhöhung der Biosynthese-Kapazität gesteigert. Dies zeigt, dass die Monoterpene - neben ihren ökologischen Funktionen – auch an der Dissipation des massiven Energie-Überangebots unter Trockenstress beteiligt sind.

Contents

Abbreviations

APS	Ammonium peroxydisulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
C	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
DTT	1,4-dithiothreitol
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EST	Expessed sequence tag
F.W.	Fresh weight
g	gramme
GC	Gass chromatography
h	hour
IPTG	Isopropyl--D-thiogalactopyranoside
kD or kDa	Kilo Dalton
LB	Luria broth
m	milli
M	Molar
MCS	Multiple cloning site
MS	Mass spectroscopy
min	minute
ml	milliliter
mRNA	Messenger RNA
Ni-NTA	nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolution per minute
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
s	second
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TEMED	N,N,N`,N`-tetramethylethylenediamine
Tm	Melting temperature (primer)
Tris	Tris(hydroxymethyl)aminomethane

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic
E	Glu	Glutamic
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

I. Introduction

Secondary plant products reveal a high significance in human daily life. They determine the aroma and flavor of our food and in medicine they are used as herbal remedies or as bases for new drug development. Moreover, they are essential for perfumes , gums, resins, cosmetics, dyes, food additives, and natural pesticides (Raskin *et al*., 2002; Vanisree, 2004; Wink, 2010). For all these applications high quality of the corresponding plant materials is required. Accordingly, the quality enhancement of these drugs, i.e. the increase of the amounts of relevant natural compounds or shifts in their composition, is of special interest. In particular any modifications in the cultivation conditions for deliberately changing the secondary metabolite composition are relevant. In this context, the impact of various environmental factors on secondary metabolism represents a promising approach (Selmar, 2008). Yet, all corresponding efforts to increase the product quality require a comprehensive knowledge on the regulative pathways of secondary plant products. We all know that medicinal plants which were grown in semi-arid regions, as found in the Mediterranean region, reveal much higher concentrations of relevant natural products than equivalent plants, but cultivated in moderate climates. These differences in the contents of relevant plant secondary metabolites are primarily referred to the differences in the growing conditions of the corresponding plants, i.e., higher levels of drought stress and much higher light intensities than Central Europe. Selmar and Kleinwächter (2013) have attempted to draw fine coherences between changes in the metabolic status under stress and enhancement of natural products. The authors pointed out that as the stress induced stomata closure markedly decreases the uptake of CO_2. As a result, the consumption of reduction equivalents (NADPH + H^+) for the CO_2-fixation via calvin cycle declines considerably, generating a massive oversupply of NADPH + H^+. As a consequence, metabolic processes are pushed toward the synthesis of highly reduced compounds, like isoprenoids, phenols or alkaloids.

In order to get solid information on this topic if indeed drought stress enhances the synthesis of secondary plant products, reliable molecular stress markers for drought stress are required. In this context, dehydrins are of special interest.

Dehydrins are characterized as group II of LEA protein (Late Embryogenesis Abundant). Their up regulation have been shown under different environmental stresses (e.g., water deficit, high and low temperatures, salinity etc.) Or in responsive to the phytohormone abscisic acid (ABA; Ingram and Bartels, 1996; Allagulova *et al*., 2003; Bray, 2004; Wahid and Close, 2007). In

addition, they are considered as a class of intrinsically unstructured proteins (disordered proteins). As consequence of this characteristic, dehydrins have a high flexibility to change their structure from the disordered state to the ordered one as soon as they interact with their biological partners, such as proteins, DNA or metal ions (Hara, 2010). Therefore, it is assumed that dehydrins have various functions during cell dehydration such as cell membrane structure stabilization, radical scavenging and cryoprotective activity (Ingram and Bartels, 1996; Hara, 2010).

Dehydrins have been reported to be present in most plant cell compartments such as cytoplasm, nucleus and mitochondria, plasmodesmata. In some cases, they are associated with the plasma membrane (Godoy *et al*., 1994; Houde *et al*., 1995; Egerton-Warburton *et al*., 1997; Danyluk *et al*., 1998; Rinne *et al*., 1999; Borovskii *et a*l., 2000; Karlson *et al*., 2003). In spite of the widespread occurrence and abundance of dehydrins during cell dehydration, their physiological relevance and biochemical role are not fully understood.

In this investigation to elucidate the impact of drought stress on the secondary metabolism, common sage (*Salvia officinalis* L., Lamiaceae) was used as a model plant. Since, it is considered as one of the most important medicinal and aromatic plants with antioxidant, antimicrobial, spasmolytic, astringent and anhidrotic properties (Demirci *et al*., 2003; Perry *et al*., 2003). Furthermore, when sage had been cultivated under drought stress, the concentration as well as the overall content of monoterpenes were markedly higher than in the corresponding well watered control plants (Nowak *et al*., 2010). The GLC-analyses revealed that the sage plants used for the experiments of this study belong to the major group of S. *officinalis* plants, in which the three cyclic monoterpenes cineole, camphor and α / β -thujone are predominant and account for more than 95% of the total monoterpene amount (Selmar and Kleinwachter, 2013). The corresponding key enzymes for monoterpene biosynthesis are the cineole synthase for 1,8 cineole, the bornyl synthase for camphor and the sabinene synthase for thujone (Wise *et al*., 1998; Selmar and Kleinwachter, 2013). Up to now, no data on the expression of monoterpene synthases under drought stress are available. However, the cDNAs encoding these three monoterpene synthases already had been successfully isolated and functionally expressed in *Escherichia coli* (Wise *et al*., 1998). As outlined above, these coherences have not effectively been addressed so far; therefore these investigations are aimed to shed light on this topic.

Strategies and objectives:

This investigation was aimed to contribute to the basic understanding of the complex interactions between the so-called stress metabolism and the secondary metabolism in general, and the synthesis and accumulation of monoterpenes in particular. Using *Salvia officinalis* as model plant, both stress and secondary metabolism have been analyzed. These data provide important information to enhance the quality of remedies derived from medicinal plants and will contribute to an improvement of the agricultural cultivation of medicinal plants in Egypt.

To elucidate this complex metabolic situation, a reliable molecular stress marker for drought stress was required. Accordingly, the objectives were addressed through the following:

1- Isolation and characterization of dehydrin gene from *Salvia officinalis.*

2- To elucidate if the dehydrin gene expression is a reliable molecular stress marker for drought stress in *Salvia officinalis.*

3- Monitoring the occurrence of dehydrin expression at protein-level.

4- Determination of monoterpene contents in sage under different drought stress conditions.

5- To demonstrate if the expression levels of monoterpene synthases are altered in response to drought stress.

II. Scientific Background

The major goal of this investigation is to increase the understanding of the complex interactions between the so-called stress metabolism and the secondary metabolism in plants in general. In particular, the impact of drought stress on dehydrin expression and the synthesis and accumulation of monoterpenes was analyzed using sage (*Salvia officinalis*) as a model plant. In this context, the applicability of dehydrins as molecular markers for drought stress was studied and evaluated. In the following chapters the current scientific literature on the various aspects of dehydrins, drought stress and secondary metabolism is reviewed.

1. Dehydrins (Dehydration proteins) in plants

Dehydrins (DHN) are a group of highly abundant proteins ranging in size from 12 to 200 kDa. In principle, dehydrins are characterized as group II of highly hydrophilic proteins known as LEA (Late Embryogenesis Abundant) in higher plants. Dehydrins are up regulated under environmental stresses (i.e., water deficit, high and low temperatures, salinity etc.; Ingram and Bartels, 1996; Bray, 2004; Wahid and Close, 2007). Sometimes, dehydrins also are termed as RAB proteins (Responsive to ABA), since their expression is increased by the phytohormone abscisic acid (ABA) (Parra *et al*., 1996; Choi *et al*., 1999; Hundertmark and Hincha, 2008).
In spite of the widespread occurrence and abundance of dehydrins during cell dehydration, their physiological functions and biochemical role are not fully understood.

1.1. Dehydrin structure, properties, and classification

Dehydrins are classified according to the occurrence of highly conserved sequence motifs, termed as K-, S-, and Y-segment (Figure 1; (Close, 1996). The K-segment is a conservative lysine-rich motif of about 15 amino acids, i.e. EKKGIMDKIKEKLPG or related sequences. It is essential for all dehydrins and usually occurs near the C-terminus (Close, 1996, 1997). The other distinctive dehydrin feature is denoted as S-segment exhibiting a track of serine residues (LHRSGSSSSSSEDD or similar sequences; Rorat, 2006). The last typical dehydrin feature, the Y-segment, which presents in the N-terminus of the protein (T/VDEYGNP; Allagulova *et al*., 2003; Rorat, 2006). In addition to these three conservative motifs, some dehydrins also reveal a

less conservative domains consisting of Gly and polar amino acids named ø-segment (Close *et al*., 1989; Close, 1996).

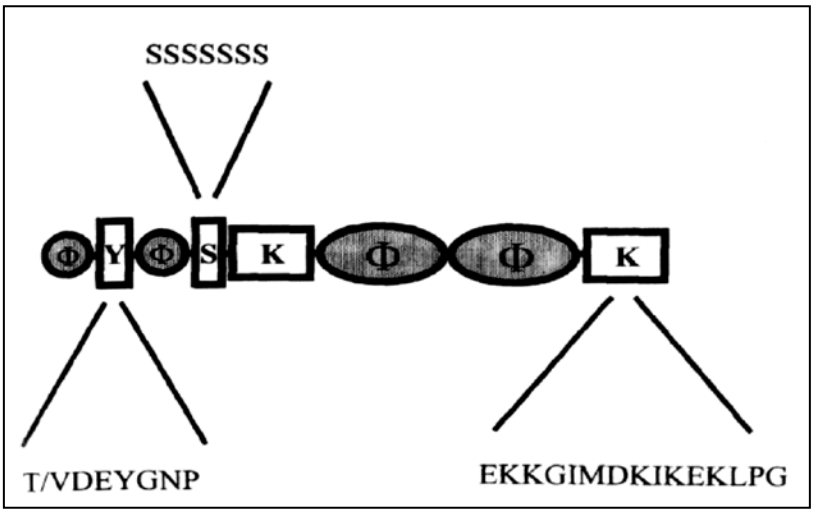


Figure 1: Structure of maize seed dehydrin YSK_2, (accessions 82684 and100918; Close, 1997).

Close (1996) sub-classified dehydrins on the basis of the numbers and order of the conservative domains K-, S-, Y-segments into five subclasses: Y_nSK_2, Y_2K_n, K_n, K_nS, and SK_n (where n is the number of repeats).

Interestingly, the regulation of these sub-classes is not uniform. Moreover, it is assumed that each sub-class reveals different functions, since their expression differs in response to various environmental factors (Rorat, 2006). For instance, the basic Y_nSK_2 type is induced by drought and/or ABA but not by low temperatures. In contrast, the synthesis of acidic dehydrins of Y_2K_n , K_n, K_nS, and SK_n types is induced favorably in response to low temperature (Allagulova *et al*., 2003; Battaglia *et al*., 2008; Hanin *et al*., 2011).

1.2. Putative functions of dehydrins

Dehydrins also are considered as a class of Intrinsically Unstructured /Disordered Proteins (IUPs/ IUDPs; Mouillon *et al*., 2006; Eriksson *et al*., 2011; Hanin *et al*., 2011). In contrast to most proteins exhibiting a classical protein structure-function relationship, the function of these proteins does not depend on their 3-D structure (Tompa, 2005). The function of many IUPs/ IUDPs is associated with the regulation of gene expression, cellular signal transduction, and protein chaperones (Figure 2); (Wright and Dyson, 1999; Tompa and Csermely, 2004; Dyson and Wright, 2005; Tompa, 2005).

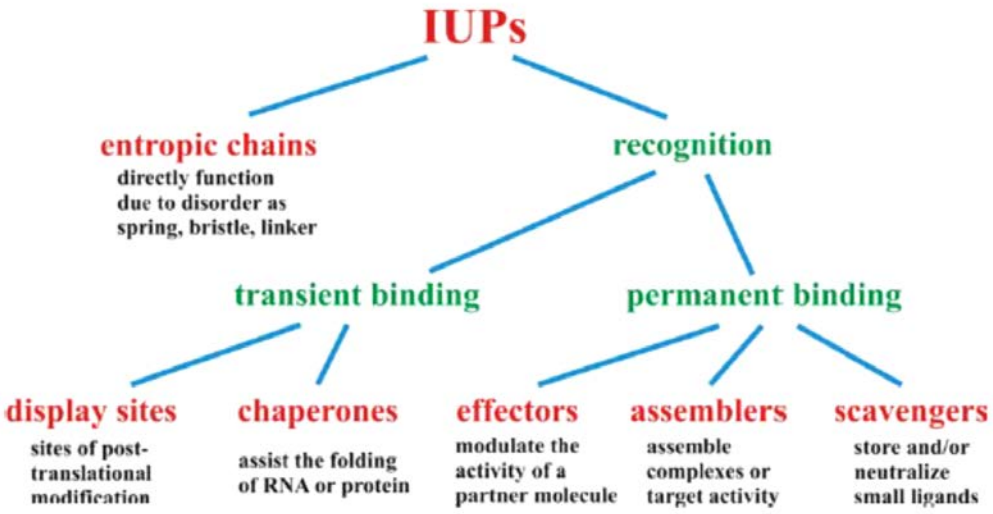


Figure2: Putative function of Intrinsically Unstructured Proteins (IUPs) according to Tompa, (2005).

Hara (2010) proposed that as consequence of the lack of typical well-structured protein features of the dehydrins, they have a high flexibility to change their conformation according to the surrounding microenvironment from the disordered state to the ordered one as soon as they interact with their biological partners, such as proteins, DNA or metal ions (Figure 3). Accordingly, this binding induces folding and thereby activates the function of the dehydrins.

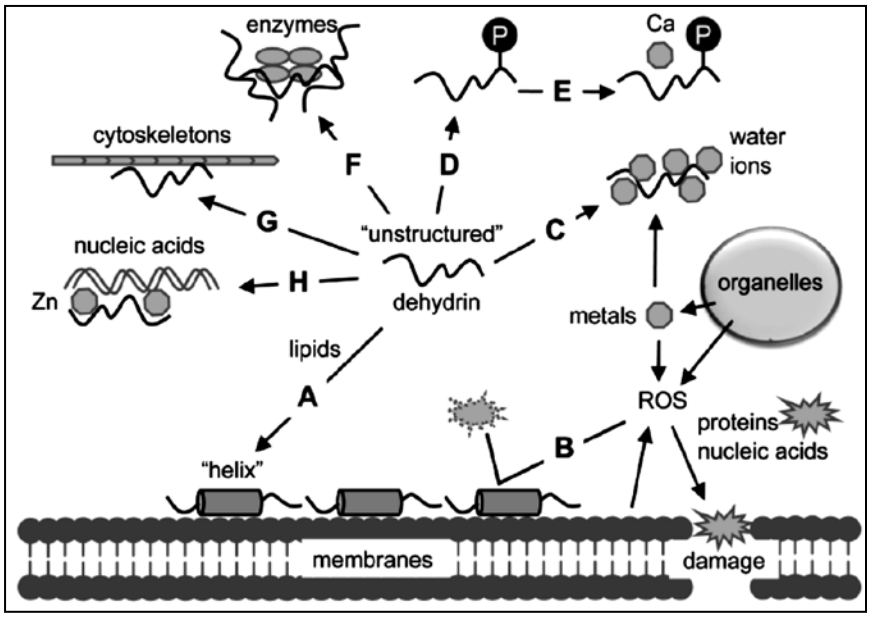


Figure 3: Putative functions of dehydrins according to Hara (2010).

A: binding to phospholipids; B: radical scavenging; C: binding to water and ions; D: phosphorylation; E: binding to calcium; F: protection of enzymes; G: binding to cytoskeletons; H: binding to nucleic acids.

1.2.1. Stabilization of cell membrane structure

A common feature of all dehydrins is the conserved motif k-segment. This unique motif bears a resemblance to a lipid-binding class A2 amphipathic α-helical segment found in apolipoproteins (one of the plasma lipoprotein components). Their putative function is related to a facilitation of the transport of water-insoluble lipids into the plasma (Close, 1996; Velten and Oliver, 2001). In analogy, dehydrins also might act at the interface between membrane phospholipids and cytosol in plants. (Campbell and Close, 1997). These authors also proposed as further analogy that once protoplasmic water activity drops; dehydrins may expose hydrophobic patches on polypeptides to prevent protein-protein aggregation at the surface in a manner similar to the well-studied model of heat shock proteins (Hsps) chaperone machines. In this case, the DnaJ and DnaK (both Hsp70) and GroEL (Hsp60) chaperones recognize their polypeptide substrates via hydrophobic interactions (Campbell and Close, 1997)

Immunolocalization studies showed that dehydrins are localized in the cell membrane, suggesting that they may play a role in cell membrane structure stabilization under dehydration (Danyluk *et al*., 1998; Koag *et al*., 2009).

Koag and his research team (2009) performed an elegant experiment to consider whether the K-segment is involved in cell membrane stabilization: using *in vitro* site directed mutagenesis, three K-segment deletion mutants forms of Maize (*Zea mays*) DHN1 had been produced. The results revealed that the K- segment is essential for the binding between acidic phospholipid vesicles and DHN1. Moreover, the acidic phospholipid vesicles stimulated the helicity of DHN1 K-segment. Beside the K-segment, the ø-segment is also thought to increase the hydrophilicity of dehydrin structure, since it reveals a high ratio of polar amino acid residues. These might contribute to the hydrophilic interactions between dehydrins and polar groups of macromolecules and between dehydrins and low molecular weight components of nucleoplasm and cytosol, such as sugars and amino acids. Taken together, the K- and ø-segment stabilize the cell structure via preventing further aggregation of partially denatured macromolecules under unfavorable conditions (Campbell and Close, 1997; Kosova *et al*., 2007)

1.2.2. Cryoprotective activity

Different dehydrins have been shown to be involved in the cryoprotection of lactate dehydrogenase (LDH) from freezing and thawing damage (Kazuoka and Oeda, 1994; Momma *et al*., 2003; Peng *et al*., 2008; Hughes and Graether, 2011). It has been reported that COR15 from

Arabidopsis exhibits cryoprotective activity that is about 10^6 times more effective than that of sucrose and 10^2-10^3 times more potent than that of BSA (Lin and Thomashow, 1992). while, Wisniewski *et al*. (1999) found that the cryoprotective and antifreezing activity of a dehydrin from peach PCA60 (*Prunus persica*) is less than that of COR15. In transgenic tobacco (*Nicotiana tabacum* L.), a K_3S type dehydrin from Citrus (*Citrus unshiu Marcov*.) enhanced the cold tolerance. The authors also reported that a dehydrin from citrus heterologously expressed in *E. coli* reduced peroxidation of soybean (*Glycine max* L.) liposomes *in vitro.* The inhibitory activity of this dehydrin against liposome oxidation was more potent than that of proline, glycine, albumin, glutathione, betaine, and sucrose (Hara *et al*., 2003).

Another citrus dehydrin CuCOR19 showed radical scavenging activity for hydroxyl and peroxyl radicals. CuCOR19 was more effective than mannitol and serum albumin, which is known as an antioxidative protein in mammals (Hara *et al*., 2004).

1.2.3. Metal binding ability

Cell dehydration leads to ion leakage from organelles and membranes. In consequence, the concentration of intracellular free metal ions increases, causing oxidative damages. It has been illustrated that citrus dehydrin (CuCOR15) binds to free metal ions Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} but does not bind to Mg^{2+}, Ca^{2+}, and Mn^{2+}. Remarkably, the highest affinity was detected for Cu^{2+}. Up to 16 Cu^{2+} ions bind to one CuCOR15. Using Immobilized Metal Ion Affinity Chromatography (IMAC), it was shown that the histidine residues are involved in the Cu^{2+} dehydrin binding. This study suggests that the binding of metals ions to CuCOR15 is due to a specific histidine containing sequence. Moreover, citrus dehydrin seems to act as radical-scavenging protein, and thereby may reduce metal toxicity in plant cells under stress conditions (Hara *et al*., 2005).

More direct evidence for the ability of dehydrins to bind metal ions was elaborated by Mu *et al*. (2011). Using Circular Dichroism (CD) spectra analysis and thermal stability assays, these authors demonstrated that dehydrin (MpDhn12) binds Cu^{2+} *in vitro* as well as *in vivo.* Xu and his co-workers (2008) provided *in vivo* evidence that the overexpression of SK_2-type dehydrin gene (BjDHN2/ BjDHN3) enhances the tolerance against Cd and Zn. The SK_2-type dehydrin isolated from the heavy-metal hyperaccumulator *Brassica junce* had been introduced in tobacco. As consequence, electrolyte-leakage level was suppressed and the content of malondialdehyde - a decay product of unsaturated fatty acids that sometimes is used as marker for oxidative stress -

decreased. From this it was deduced that the tolerance against heavy metals is improved by BjDHN2/BjDHN3 by attenuating lipid peroxidation and by protecting membrane structure.

Krüger *et al*., (2002) suggesting that the crucial function of dehydrins may be related to iron transport similar to the ITP (iron transport protein) from castor bean (*Ricinus communis* L.) which transports ions Fe^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}, since these highly reactive species require masking of their electrical charge in the cytoplasm and phloem.

1.3. Distribution of dehydrins in plant tissues

The widespread distribution of dehydrins in various plant parts implies that it has a high relevance. It is thought that dehydrins reveal many physiological functions, especially with respect to metabolic responses to various environmental stresses, but also under ordinary conditions (Allagulova *et al*., 2003; Hara, 2010). The localization of four dehydrin (RAB18, ERD14, LTI30 and LTI29) proteins accumulated in *Arabidopsis* was analyzed immunologically by Nylander *et al*. (2001) using specific antibodies. In unstressed plants, dehydrin RAB18 was localized in stomatal guard cells, ERD14 occurred in the vascular tissue and adjacent parenchymal cells, and LTI29 and ERD14 accumulated in the root tips. LTI30 was not detected in unstressed plants. In contrast, in stressed plants, the localization of LTI29, ERD14 and RAB18 was not restricted to certain tissues or cell types but it was accumulated in most cells. Intracellular localization of dehydrins seems to vary: numerous studies revealed that dehydrins are localized in the cytoplasm, the nucleus, the mitochondria, the plasmodesmata and sometimes they are associated with the plasma membrane (Godoy *et al*., 1994; Houde *et al*., 1995; Egerton-Warburton *et al*., 1997; Danyluk *et al*., 1998; Rinne *et al*., 1999; Borovskii *et al*., 2000; Karlson *et al*., 2003)

Some dehydrins are mainly located in mature seeds, for instance in *Zea mays* the dehydrins RAB17 and DHN1 are located in all parts of the embryo as well as in the endosperm of mature seeds (Goday *et al*., 1988). Another 26-kDa dehydrin was purified from soybean (*Glycin max*) mature seeds (Momma *et al*., 1997). Using immunohistochemistry and *in situ* hybridization, dehydrin from carrot was detected to be located in the endosperm and zygotic embryos of mature seeds (Kiyosue *et al*., 1993). It turned out that dehydrins are present in all orthodox seeds, whereas they generally are absent in recalcitrant seeds. The reason for this difference in the distribution of dehydrins is due to the presence or absence of a maturation drying. All orthodox

seeds pass through this developmental phase, which is the basis for seed dormancy, enabling the seeds to withstand unfavorable wintery conditions. Accordingly, dehydrins in seeds had been denoted as late embryogenesis abundant proteins (LEA-proteins). In contrast, in typical recalcitrant seeds this protective mechanism is not required, since there are no severe seasonal changes in the tropics, which have to be outlasted by the seeds. This may be the reason, why, in recalcitrant seeds, maturation drying is not part of the standard seed development (Figure 3); (for detailed information see Radwan *et al*., 2014). A profound conformation that indeed differences in maturation drying are responsible for the differential expression of dehydrins is given by intermediate seeds, such as coffee seeds (*Coffea arabica*). Like recalcitrant seeds coffee seeds do not pass a maturation drying (Kramer *et al*., 2010); accordingly they do not express dehydrins during embryogenesis. However, if these seeds are dried artificially, dehydrins are expressed and the seeds behave like orthodox seeds, i.e. they could be stored without loosing their viability. Another group of seed, the so-called atypical recalcitrant seeds, such as those from oak (*Quercus robur*) or chestnut (*Aesculus hippocastaneum*), which show just a faint maturation drying (Finch-Savage *et al*., 1992; 1994) indeed express dehydrins. However, the corresponding expression is far lower than in orthodox seeds and, accordingly, these seeds relative rapidly loose their viability (Figure 4).

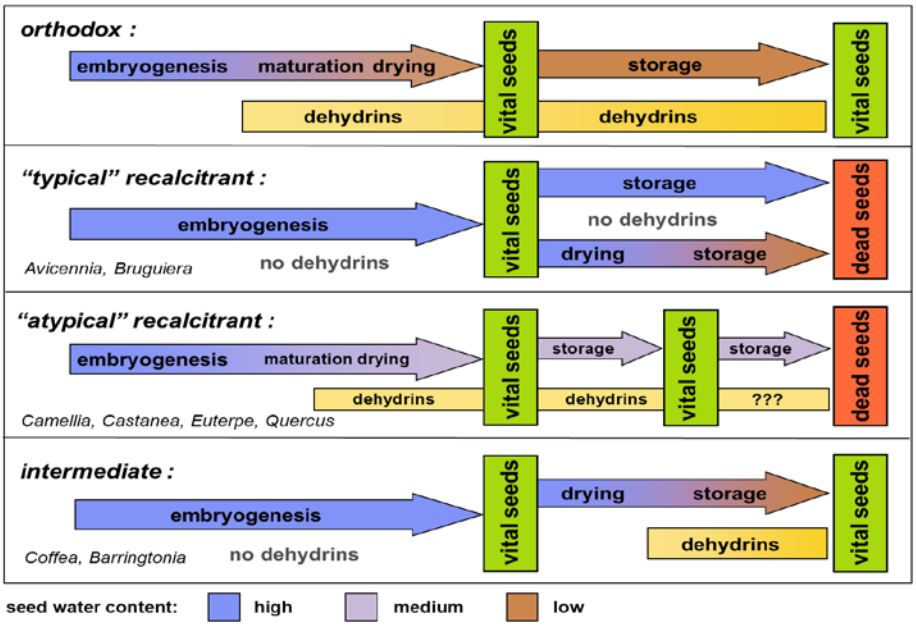


Figure 4: Dehydrins occurrence in seeds during maturation drying stages according to Radwan *et al*. (2014)

2.4. Posttranslational modification

The most abundant posttranslational modification of dehydrin proteins is the phosphorylation (Hanin *et al*., 2011)**.** In early studies, Vilardell *et al*. (1990) reported that maize dehydrin RAB-17, which is responsive to abscisic acid, has a potential phosphorylation site. This site is a cluster of serine residues (S-segment) followed by a putative casein-type kinase 2-type substrate consensus sequence. Another study showed that *in vitro* as well as in *in vivo* experiments the RAB-17 is phosphorylated by casein kinase 2 and this phosphorylation occurs in the S-segment (Plana, 1991)**.** Jensen *et al*. (1998) proposed that phosphorylation mediates the translocation of dehydrin from the cytoplasm, the site where it is synthesized, into the nucleus. In addition, these authors identified the stretch from amino acid residues 66 to 96, which contains the S-segment and which is adjacent to the protein kinase CK2 recognition site and a sequence of basic amino acids residues (RRKK) sharing similarity to a nuclear localization signal (NLS) of a simian virus 40 (SV40) signal peptide. Further studies on dehydrins from arabidopsis (*Arabidopsis thaliana*), celery (*Apium graveolens*) and tomato (*Lycopersicon esculentum*) confirmed that these proteins indeed are phosphorylated (Godoy *et al*., 1994; Heyen *et al*., 2002; Alsheikh *et al*., 2003).

In contrast, in the dehydrin family WCS120 from wheat the S-segment and the protein kinase recognition site are lacking. Therefore, it is proposed that the wheat dehydrins are co-translocated into the nucleus via protein–protein interactions (Sarhan *et al*., 1997).

2.5. Regulation of dehydrin expression

The complex differences in dehydrin expression under environmental stresses as well as even under ordinary conditions indicates the existence of a various different regulatory pathways (Allagulova *et al*., 2003). Evidences indicated that DHN is up regulated by the phytohormone abscisic acid (ABA) (Giordani *et al*., 1999; Lee *et al*., 1996; Olave-Concha *et al*., 2004; Parmentier-Line *et al.*, 2002). Giordani *et al*. (1999) investigated the relationship between abscisic acid (ABA) and the accumulation of dehydrins using ABA-deficient mutants of sunflower (*Helianthus annuus L*.). These authors conclude that the regulation of dehydrin gene expression occurs via two pathways: ABA-dependent and ABA-independent pathways.

Yamaguchi-Shinozaki and Shinozaki (1994) identified specific regulatory sites for ABA-independent pathway and named dehydration-responsive element (DRE). These sites correspond

to *cis*-acting elements, containing 9 bp (TACCGACAT), which were found in the promoter of the dehydrin *rd29a* gene, which is expressed in response to dehydration, salinity, and cold. Other elements had been characterized by Stockinger *et al*. (1997), as C-repeat/DRE binding factor from *Arabidopsis thaliana.* These factors stimulate the transcription activity in response to water deficit and low temperature.

While the ABA-dependent pathway refers to the presence of potential *cis*-acting DNA elements, known as ABA responsive elements (ABRE) in the gene promoter region (Guiltinan *et al*., 1990; Shinozaki and Yamaguchi-Shinozaki, 1997). These elements contain the G-box (TACGTCC) and the GC motif (GGCCGCG), which both belong to the basic leucine zipper protein transcription factors (Leung and Giraudat, 1998; Kang *et al*., 2002; Seki *et al*., 2003).

2. Relevance of plant secondary metabolites

Secondary metabolites are known as compounds revealing no-fundamental role in the primary metabolism of plants. In contrast, they have a great significance for the interaction with their environment and related adaptation processes. In this context, defense and competition on the one hand and stimulation and attraction on the other hand are of special importance. In addition, all responses of the plants related to various stresses impact on or at least are linked to

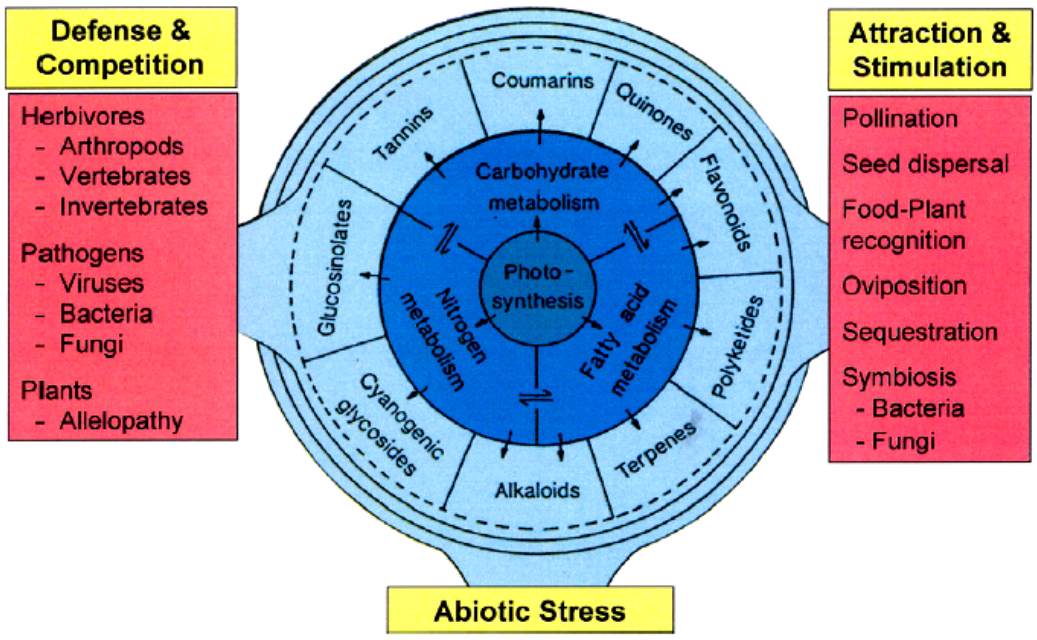


Figure 5: Ecological relevance of plant secondary metabolism under different stressors (Hartmann, 2007)

the plant's secondary metabolism (Figure 5 according to Hartmann, 2007; for review see e.g. Bennett and Wallsgrove, 1994; Harborne, 1990; Ramakrishna and Ravishankar, 2011).

Beside the biological significance of plant secondary metabolites, they also have a great commercial and economical importance. They are used as medicine, as basis for new drug development, as flavors, gums, resins, cosmetics, natural rubber, dyes, fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988; Vanisree, 2004).

2.1. Ecological function of plant secondary metabolites

Whereas in the last century plant secondary metabolites have been considered as waste compounds, meanwhile numerous studies have elucidated their important ecological significance, especially with respect to their role in plant protection against herbivores, pathogens but also against abiotic stresses (Poecke *et al*., 2001; Hartmann, 2004; Heil and Bueno, 2007). The interactions between pathogens and secondary metabolites have been thoroughly studied, e.g. Moreno-Osorio *et al*. (2008) tested the toxic effects of polygodial and its acetal, propylene and ethylene derivatives on some insect species, such as *Spodoptera littoralis*, *Leptinotarsa decemlineata, Myzus persicae* and *Rhopalosiphum padi*. They found that these constituents have a strong repellent activity. Many other studies reported that pyrrolizidine alkaloids as a typical compounds of plant secondary metabolism are constitutively produced as a defense against insect and vertebrate attack (Hartmann and Ober, 2000; Pasteels *et al.*, 2001; Anke *et al*., 2008). Other studies elucidated the role of glucosinolates in plant's defense against herbivores and pathogens (for detailed information see e.g. Mewis *et al*., 2002, 2005; Martin and Müller, 2007).

In parallel to the impacts of natural products on herbivores and pathogens also the interactions between plant secondary metabolites and environmental stresses were investigated. A prospective study on the impact of drought stress on secondary metabolism is presented by (Leopoldini *et al*., 2006). The authors showed that the flavonoid quercetin has an antioxidant capability by iron chelation. Another study proposed that isoprene reveals a protective function as putative antioxidant, which prevents the photosynthetic apparatus from destruction through quenching of H_2O_2, which is formed by photosynthesis when stomata are closed in *Phragmites australis* (Loreto and Velikova, 2001). Many others studies stated that polyamines (PAs) such as (spermine, spermidine and putrescine) have abilities to stabilize membranes and the cell wall via positive charges in polyamines that allow electrostatic interactions with negatively charged loci

in macromolecules as DNA, RNA, phospholipids, or certain proteins (Berta *et al*., 1997; Martin-Tanguy, 2001; Gill and Tuteja, 2010).

There is no doubt that plant secondary metabolites reveal a high importance to protect against biotic and abiotic stresses. Yet, the particular impact of drought stress on plant secondary metabolism is very often neglected. Therefore, in the following section, the relevance and interference of plant secondary metabolism with respect to drought stress is outlined in detail.

2.2. Drought stress correlated to plant secondary metabolism

Many studies showed that plant secondary metabolites are accumulated under drought stress (for review see Selmar and Kleinwächter, 2013). The authors outline that the concentration of most classes of secondary metabolites strongly is enhanced by drought stress. This accounts for simple and complex phenols, terpenes and essential oils as well as for alkaloids and other nitrogen-conaining compounds, such as glucosinolates and cyanogenic glycosides (Table 1). An emblematic example is the massive increase in the concentration of total soluble phenols and complex phenols such as 1.5-dihydroxyxanthone, quercetin and rutin in the medicinal plant (*Hypericum brasiliense*), (De Abreu and Mazzafera, 2005). In the same manner, Bettaieb *et al*. (2009) reported a strong increasing of essential oils concentration in *Salvia officinalis* under drought stress as well as the concentration of quinolizidine alkaloids also is increased under drought stress (Christiansen, *et al*., 1997). Analogously, Jensen *et al*. (1996) found a massive increase in the concentration of glucosinolates in response to soil drying and also trigonelline was reported to increase in *Glycine max* in response to water deficit (Cho *et al*., 2003). In addition, in drought stressed coffee seeds (*Coffea arabica* L.) the concentration of γ- amino-butyric acid (GABA) increases tenfold in comparison to the unstressed controls (Bytof *et al*., 2005).

However, increasing concentrations of secondary metabolites in plants submitted to drought stress, does not necessarily mean that in the corresponding plants the biosynthesis of secondary metabolites also is enhanced: As drought stressed plants generally reveal growth rates that strongly are diminished, they frequently produce significantly less biomass than the well-watered controls. Accordingly, assuming that in both plants the rate of natural product biosynthesis is similar and thus the total amounts equal, as result, the concentration of natural products (e.g. amount per gram fresh or dry weight) will increase; i.e. the concentration increase

	Simple Phenols		
Helianthus annuus	chlorogenic acid	massive increase (tenfold)	del Moral 1972
Prunus persica	total phenols	higher contents	Kubota et al. 1988
Thymus capitatus	phenolics	higher contents	Delitala et al. 1986
Echinacea purpurea	total phenols	strong increase (67 %)	Gray et al. 2003
Crataegus spp.	chlorogenic acid	massive increase (2 - 6fold)	Kirakosyan et al. 2004
Hypericum brasiliense	total phenols	strong increase (over 80 %)	de Abreu et al. 2005
Trachyspermum ammi	total phenols	strong increase (100 %)	Azhar et al. 2011
Labisia pumila	total phenols	significant increase (50 %)	Jaafar et al. 2012
	Complex Phenols		
Pisum sativum	flavonoids	strong increase (45 %)	Nogués et al. 1998
Pisum sativum	anthocyanins	strong increase (over 80 %)	Nogués et al. 1998
Crataegus spp.	catechins / epicatechins	massive increase (2 - 12fold)	Kirakosyan et al. 2004
Hypericum brasiliense	rutine / quercetin	massive increase (fourfold)	de Abreu et al. 2005
Hypericum brasiliense	xanthones	strong increase (over 80 %)	de Abreu et al. 2005
Camellia sinensis	epicathecins	higher contents	Hernández et al. 2006
Salvia miltiorrhiza	furoquinones	significant increase	Liu et al. 2011
Prunella vulgaris	rosmarinic acid	slight increase	Chen et al. 2011
Labisia pumila	anthocyane / flavonoids	significant increase	Jaafar et al. 2012
	Monoterpenes / Essential Oils		
Mentha x piperita ssp.	essential oils	significant increase	Charles et al. 1990
Cymbopogon pendulus	geraniol & citral	strong increase	Singh-Sangwan et al.1994
Pinus halepensis	α-pinen, carene	strong increase	Llusià and Peňuelas 1998
Cistus monospeliensis	caryophyllene	enormous increase	Llusià and Peňuelas 1998
Satureja hortensis	essential oils	increase	Baher et al. 2002
Picea abies	monoterpenes	strong increase	Turtola et al. 2003
Pinus silvestris	monoterpenes	strong increase	Turtola et al. 2003
Petroselinum crispum	essential oils	strong increase (double)	Petropoulos et al. 2008
Salvia officinalis	essential oils	massive increase (2 - 4fold)	Beattaieb et al. 2009
Salvia officinalis	monoterpenes	strong increase	Nowak et al. 2010
Scrophularia ningpoen.	iridoid glycosides	increase	Wang et al. 2010
Nepeta cataria	essential oils	significant increase	Manukyan 2011
Ocimum basilicum	essential oils	significant increase	Forouzandeh et al. 2012
	Di- and Triterpenes		
Solanum tuberosum	steroid alkaloids	strong increase	Bejarano et al. 2000
Hypericum brasiliense	betulinic acid	strong increase	de Abreu et al. 2005
Bupleurum chinense	saikosaponin	significant increase	Zhu et al. 2009
Prunella vulgaris	triterpenes	slight increase	Chen et al. 2011
	Alkaloids		
Senecio longilobus	pyrrolizidine alkaloids	strong increase	Briske and Camp 1982
Lupinus angustifolius	quinolizidin alkaloids	strong increase	Christiansen et al. 1997
Solanum tuberosum	steroid alkaloids	strong increase	Bejarano et al. 2000
Glycine max	trigonelline	higher contents	Cho et al. 2003
Papaver somniferum	morphine alkaloids	strong increase	Szabó et al. 2003
Catharanthus roseus	indole alkaloids	strong increase (with Ca^{2+})	Jaleel et al. 2007
Phellodend. amurense	benzylisoquinolines	strong increase	Xia et al. 2007
Senecio jacobaea	pyrrolizidine alkaloids	massive increase	Kirk et al. 2010
Nicotiana tabacum	nicotiana-alkaloids	strong increase	Çakir and Çebi 2010
	Various Classes		
Manihot esculenta	cyanogenic glucosides	strong increase	de Bruijn 1973
Triglochin maritima	cyanogenic glucosides	strong increase	Majak et al. 1980
Brassica napus	glucosinolates	massive increase	Jensen et al. 1996
Coffea arabica	γ-aminobutyric acid	massive increase (tenfold)	Bytof et al. 2005
Brassica oleracea	glucosinolates	significant increase	Radovich et al. 2005
Brassica carinata	glucosinolates	significant increase	Schreiner et al. 2009
Phaseolus lunatus	cyanogenic glucosides	higher content in stressed plants	Ballhorn et al. 2011

Table 1. Influence of drought stress on various classes of plant secondary metabolites content (Selmar and Kleinwachter, 2013).

just reflects the reduction in biomass production in response to drought stress and not any impact on the rate of biosynthesis (Selmar and Kleinwächter, 2013). For such considerations, the differences in the total amounts of secondary metabolites in stressed and well-watered plants have to be determined rather than the differences in concentration on fresh or dry weight basis.

Unfortunately, only in very few studies the total amount of secondary plant products in drought stressed and well-watered plants had been documented: De Abreu and Mazzafera (2005) compared between the total amounts of phenolics in *Hypericum brasiliense* in stressed and well-watered plant. The authors found that not only the concentration but also the total content of the phenolic compounds is drastically higher in plants grown under drought stress than in the control plants. A study by Nowak *et al.* (2010) revealed that the total content of monoterpenes (i.e. the product of concentration and total biomass) in sage also significantly is enhanced by moderate drought stress. In contrast to this study Manukyan (2011) mentioned that the total content of terpenoids in *Melissa officinalis, Nepeta cataria and Salvia officinalis* is decreased, although their concentration was increased.

In addition to these experimental data, there is a general well-known phenomenon that points out that drought stress positively impacts on the concentration of natural products: when spices plants are grown in semi-arid climates, where they suffer far more drought stress than under well watered conditions, they are much more aroma-intensive than those obtained from equivalent plants, but cultivated in a moderate climate (Selmar, 2008).

Despite of these well-known coherences, up to date there are only few studies available dealing with the interaction between abiotic stress and plants secondary metabolism (Selmar, 2008). A corresponding attempt was made by Selmar and Kleinwächter (2013), who focused on the impact of the over-reduced status, which results in the photosynthetic apparatus in plants exposed to drought stress. These authors highlighted the complex interactions between photosynthetic status and the corresponding reduction state on plants secondary metabolism in stressed and unstressed plants (Figure 6). Drought stress induces stomata closure and the uptake of CO_2 markedly decreases. As a result, the consumption of reduction equivalents ($NADPH + H^+$) for the CO_2-fixation via Calvin cycle declines significantly, generating a massive oversupply of $NADPH + H^+$. Accordingly, the electron transport chain produces much more electrons than could be transferred to $NADP^+$. This imbalance between the delivery and consumption of

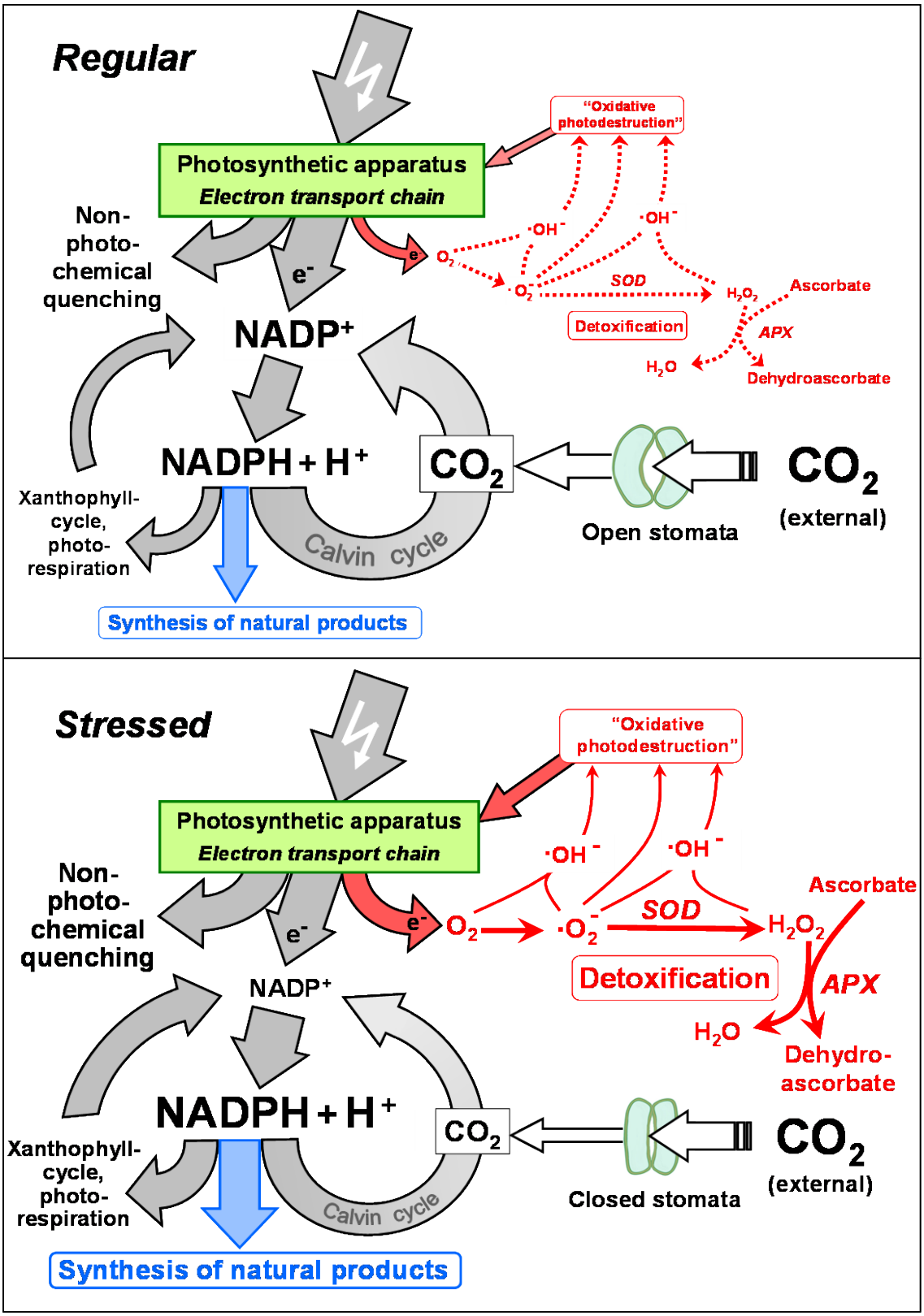


Figure 6: Plant protective mechanisms (non-photochemical quenching, photorespiration, xanthophyll cycle, superoxide dismutase (SOD), and ascorbate peroxidase (APX); (Selmar and Kleinwächter, 2013).

reduction equivalents ($NADPH + H^+$ and $NADP^+$) generates various reactive oxygen species (ROS), which might damage the photosynthetic apparatus. Accordingly, various protective mechanisms are boosted in order to dissipate the extra of energy, i.e. the non-photochemical quenching, photorespiration and the xanthophyll cycle. Although massive amounts of light energy could be dissipated by these protective mechanisms, superoxide radicals are formed. These reactive oxygen species are detoxified by the superoxide dismutase (SOD), and ascorbate peroxidase (APX) system (Asada, 2000; Alscher *et al*., 2002; Chen *et al*., 2004; Ahmad *et al*., 2008).

Despite of all these energy dissipating mechanisms, in drought stressed plants a massive oversupply of $NADPH + H^+$ is resulting. Wilhelm and Selmar (2011) postulated that the related strong reduction power should lead to an enhancement of the synthesis of highly reduced compounds, like terpenoids, phenols or alkaloids.

The over-reduced state, which is generated under drought stress indeed is directly enhancing the rate of biosynthesis of secondary plant products was demonstrated by Nowak *et al*. (2010). These authors cultivated sage plants (*Salvia officinalis*) under well-watered and under drought stress conditions as well as under ambient and strongly enhanced CO_2-concentration (700 ppm). As outlined before, drought stress induces a significant increase in the total amount of terpenes under ambient CO_2-concentrations. Yet, when the external CO_2-concentration was strongly enhanced, as result, also the internal CO_2 concentration in the leaves should increase markedly, even when the stomata are partially closed. Consequently, the extent of CO_2-fixation via Calvin cycle increases and the over-reduced state should be diminished, leading to a decline in the synthesis of monoterpenes. As expected, Nowak *et al*. (2010) indeed determined a corresponding decline in the monoterpene contents in the sage plants cultivated in elevated CO_2 atmospheres. These results strongly support the hypothesis that the biosynthesis of secondary metabolites increases under stress conditions due to the enhancement of reduction pressure.

From these findings, it could be concluded that the synthesis and accumulation of secondary plant products could be considered as a machinery to lower the oversupply of reduction power and thereby to prevent the generation of high amounts of reactive oxygen species. However, if the supplementary consumption of reduction equivalents indeed may have an actual advantage for the plant, a corresponding increase of the synthesis of highly reduced

natural products under stress conditions should not only be accomplished simply by an "overflow", but also by increasing biosynthetic capacity. In consequence, in plants well adapted to drought stress, the expression of enzymes involved in the biosynthesis of reduced natural products should be enhanced. Accordingly, a closer look at the biosynthetic corresponding enzymes is required to get further insides into the complex processes involved in the drought stress related enhancement of secondary metabolism. In order to provide the metabolic background for such approach, a brief outline of the plant terpenoid metabolism is presented in the following chapter.

2.3. Plant terpenoids: classification and biosynthesis

The terpenoids represent the largest group of plant secondary metabolites; altogether they comprise about 40,000 different structures. since all these compounds are derived from a five-carbon isoprene unit, which is assembled and modified in various ways, they often also are denoted as isoprenoids (Peters and Croteau, 2003; Roberts, 2007). Just like the other secondary metabolites, terpenoids also reveal a high significance for the interactions of plants with their environment, and many important biological functions are described. Due to their toxic or repelling properties (e.g, polygodial; sesquiterpenoid) they are potent feeding deterrents which protect the plants against herbivores; based on their antimicrobial properties they play an important role in pathogen resistance of plants (e.g., labdane-related diterpenoids). In addition, many volatile terpenoids are involved in the attraction of pollinators (e.g pulegone; monoterpenoid ketone). Apart from their manifold ecological functions, terpenoids also are part of many processes in primary metabolism; e.g. they represent important photosynthetic pigments (carotenoids), they are involved in the regulation of growth development and signal transduction processes (e.g., gibberellins; ABA;). Moreover, they are part of electron transport chain (e.g., ubi- and plastoquinone). (Rose *et al*., 1996; Prisic *et al*., 2004; Tholl, 2006; Moreno-Osorio *et al*., 2008; Ashour *et al*., 2010; Kuzuyama and Seto, 2012).

All terpeneoids represent oligomers or polymers of an isoprene unit (five-carbon monomer). Their classification is based on the condensation of the activated basic isoprenoid unit, i.e., isopentenylpyrophosphate (IPP) and dimethylallypyrophosphate (DMAPP); (Figure 7). Accordingly, they are categorized as: hemiterpenes (one isoprene unit; C_5), monoterpenes (two

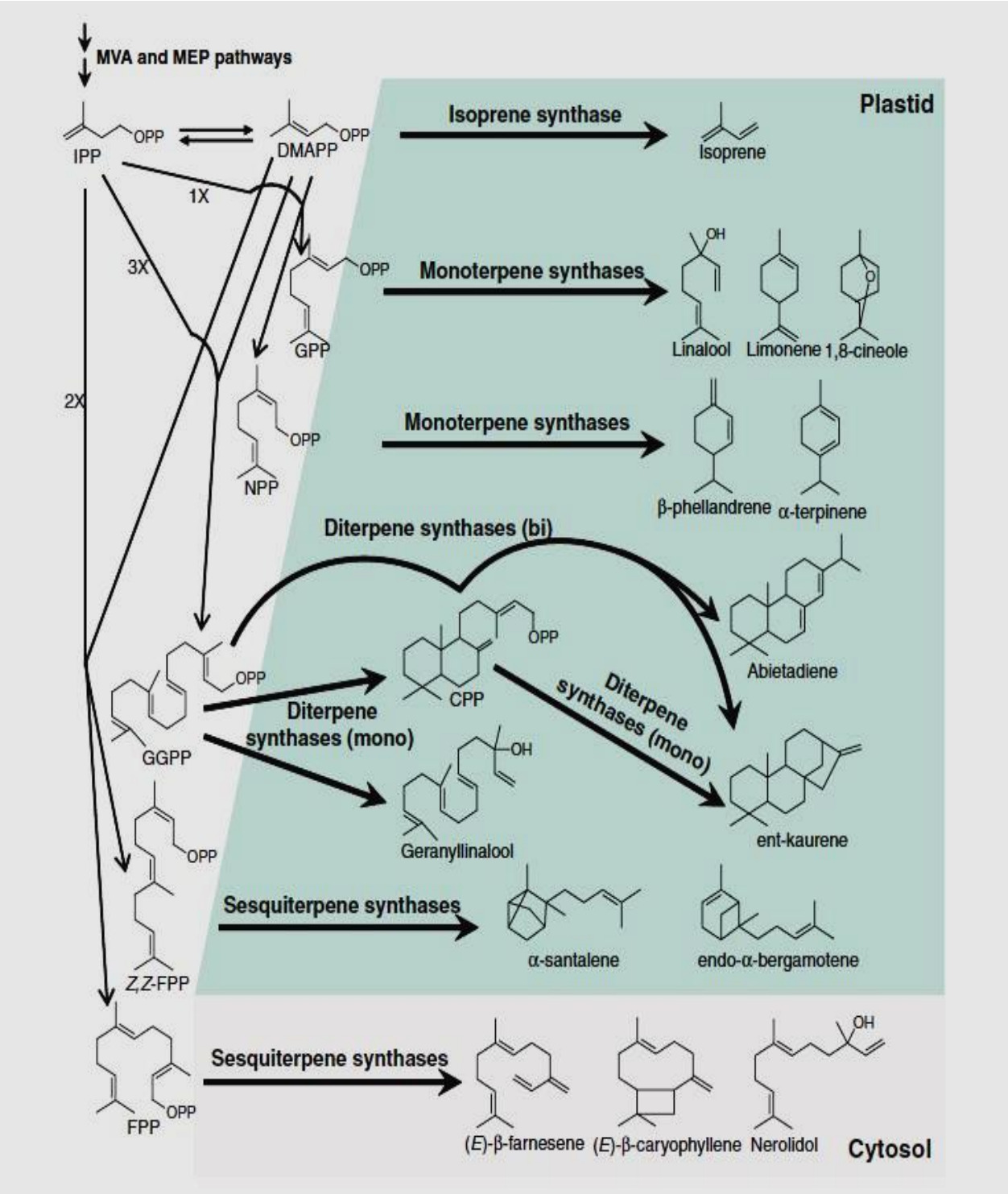


Figure 7: Overview of the terpenoid biosynthesis. The basic prcursors isopentenyldiphosphate (IPP) and dimethylallylpyrophosphate (DMAPP) are either produced via the cytosolic MVA pathway (mevalonate) or the plastidial MEP pathway (methylerythritolphosphate). GGPP: geranylgeranylpyrophosphate; GPP: geranylpyrophosphate; FPP: farnesylpyrophosphate (Chen *et al*., 2011). In recent nomenclature, instead of the term *pyrophosphate* often *diphosphate* is used.

isoprene units, ;C_{10}), sesquiterpenes (three isoprene units;; C_{15}), diterpenes (four isoprene units; C_{20}), sesterpenes (five isoprene units; C_{25}), triterpenes (six isoprenoid units; C_{30}), tetraterpenes (eight isoprene units; C_{40}) and polyterpenes (>C_{40}) (Ashour *et al*., 2010) .

The biogenesis of isoprenoids in plants takes place in different cell compartments including the cytosol, plastids, mitochondria and the endoplasmic reticulum (Lichtenthaler *et al*., 1997; Hunter, 2007). As mentioned in Figure 7, there are two distinct pathways leading to the essential metabolic precursors of isoprenoids, the so-called activated isoprenes, i.e. isopentenyl-pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP). While the mevalonic acid (MVA) pathway is located in the cytosol and the peroxisomes, the methylerythritolphosphate (MEP) pathway is operating in plastids (Bouvier *et al*., 2005; Chen *et al*., 2011). As pointed out above, all isoprenoids are synthesized by the sequential condensation of the universal five-carbon monomers IPP and its allylic isomer DMAPP. In the plastids, the activated isoprenes are derived from MEP, which is synthesized from 1-deoxy-D-xylulose 5-phosphate, via the assembing of pyruvate and D-glyceraldehyde 3-phosphate. In the cytosol and peroxisomes, the activated isoprene unit originate from the long-known, classic mevalonic acid (MVA) pathway which starts with the condensation of three acetyl-CoA units (Rohdich *et al*., 2002; Dubey *et al*., 2003; Cheng *et al*., 2007; Chen *et al*., 2011; Kuzuyama and Seto, 2012).

3. Sage (*Salvia officinalis*) as a model plant rich in monoterpenes

3.1. The derivation of the name, history and uses

The genus Salvia is the most abundant one within the Lamiaceae family and comprises more than 900 species throughout the world (Demirci *et al*., 2003). The genus *Salvia* L. is derived from the Latin word for health (*salvare*) and means to save or to heal (Kintzios, 2000). Many of Salvia species are used as herbal teas, spices, cosmetics and perfumes. Already in traditional medicine but nowadays also in pharmaceutical industry these plants are in common use due to their manifold effects, such as tonic, anti-rheumatoid, anti-inflammatory, antimicrobial and carminative properties (Demirci *et al*., 2003; Perry *et al*., 2003). Interestingly, in ancient Egypt, this herb was used to increase the fertility of women (Dweck, 2000).

Among the various sage species, *Salvia officinalis* known as common sage or garden sage (Figure 8), is the most important one. This name refers to *sauge* (sage) in French and *sawge* in

Old English. Common sage is a perennial branched shrub with woody stems used as medicinal and aromatic herb with beneficial healing properties since ancient times. Initially, this plant was

Figure 8: *Salvia officinalis*, perennial woody sub-shrub. The leaves are silvery white, heart-shaped, lumpy, and hairy. The flowers are dark blue –purple
http://en.wikipedia.org/wiki/Salvia_officinalis

native to the Mediterranean region. However, now, sage is widespread all over the world, and most abundant in three areas: in Europa around the Mediterranean, in South-East Asia, and in Central and South America (Kintzios, 2000; Mirjalili *et al*., 2006; Farkas, *et al*., 2008; Kamatou *et al*., 2008 ; Schmiderer *et al*., 2010).

Dried sage leaves with boiling water was the traditional use of sage as medicinal tea. It was well-known for the treatment of inflammations in the mouth and the throat and for relieving of excessive sweating and minor skin inflammations (Walch *et al.*, 2011). It has been suggested that sage is useful for treating sore throat, since it contains antibactericidal principles (El Astal *et al.*, 2003). Moreover, Baïracli Levy (1991) proposed that sage leaves baked together with common sea salt could be used as a tooth powder as stain remover.

Sá *et al*. (2009) studied the beneficial properties of sage tea as antidiabetic agent. The authors showed a wholesome effect after a four weeks treatment of sage tea drinking (300 mL, twice a day) by an improved lipid profile, a higher antioxidant defense and increased lymphocyte heat shock protein expression (Hsp70). In the same manner, Lima *et al*. (2005) found by *in vitro* experiment that replacement of water by sage tea (*Salvia officinalis*) for 14 days in the diet of mice and rats improves the liver antioxidant status.

Oboh and Henle (2009) demonstrated that aqueous extracts of *Salvia officinalis* leaves reveal antioxidative and inhibitory effects on lipid peroxidation induced by some pro-oxidants (like $FeSO_4$ or sodium nitroprusside) in rat brain and liver. Moreover, it has been reported that the use of sage is beneficial for the head and brain as well as it quickens the senses and memory (Eidi *et al*., 2006). An i*n vitro* experiments performed by Akhondzadeh *et al*. (2003) showed that patients with mild to moderate Alzheimer's disease had statistically significant benefits in cognition after receiving a fixed dose (60 drops/day) of *Salvia officinalis* extract for 16 weeks.

A recent research by Almeida *et al*. (2013) reported that the bicyclic monoterpene alcohol, borneol, reveals a significant central and peripheral antinociceptive activity as well as anti-inflammatory activity response in mice. Another recent research by Quintans-Júnior *et al*., (2013) evaluated the antinocicepetive and redox properties of a few of monoterpenes by conducting *in vivo* and *in vitro* experiments. They concluded that (+)-camphene, p-cymene, and geranyl acetate may show various pharmacological properties related to inflammation and pain-

related processes. Furthermore, these compounds are thought to be useful for the development of new therapeutic approaches.

On the other hand, sage drinking should be avoided during pregnancy, since it stimulates the muscles of the uterus (Dweck, 2000). Moreover, there are several hints on toxic effects of essential oil from sage. The toxicity of sage oil is thought to be caused by ketone terpenoids such as thujone and camphor. The European Parliament and Council (2008) recommended that thujone, which is naturally found in *Salvia officinalis* is not allowed to be added to food in its chemically pure form, since it causes symptoms of so-called ‘‘absinthism”, i.e. convulsions, blindness, mental deterioration and hallucinations (Lachenmeier and Uebelacker, 2010). In contrast, it is allowed as ingredients, when it is indirectly introduced into the foods by means of thujone containing plants (European Parliament and Council, 2008). The neurotoxicity of sage was investigated by Millet *et al.* (1979), who reported that in rats the sub convulsive limit dose of sage essential oil was 0.3 g/kg. Convulsions started at 0.5 g/kg and the essential oil became lethal at doses of 3.2 g/kg. The authors referred this toxicity to the occurrence of camphor, which is well-known to reveal convulsant activity.

3.2. The essential oil from *Salvia officinalis*

Nearly all natural essential oils are chemically complex mixtures. Frequently, they are composed of a large variety of ecologically relevant compounds, belonging to different chemical classes such as monoterpenoids, sesquiterpenoids and phenylpropanoids (Sangwan *et al*., 2001).

In general, essential oils are very interesting with respect to their biological activity. Moreover, their complex chemical compositions could be used to resolve certain taxonomic problems and discrepancies, especially in the Lamiaceae (Wink, 2003). These volatiles are well known as odorous, revealing a very high economic importance in perfumery, in cosmetics as well as in the pharmaceutical industry (Biswas *et al*., 2009). In leaves, they are formed and stored in special epidermal essential oil glands. In these specialized structures, terpenes are transported through the cytoplasm and the cell wall and stored in subcuticular oil storage cavities. There are two main glandular hair types in *S. officinalis*: peltate and capitate glands. Peltate glands have one basal cell, one short stalk cell, four center cells and eight periphery head cells. Capitate glands (type I–III) may reveal several stalk cells and one or two special head cells, or a group of cells such as glandular trichomes (Corsi and Bottega, 1999; Biswas *et al*., 2009; Schmiderer *et*

al., 2010). Trichomes are structures responsible for the secretion of essential oil. The main relevance of these structures is thought to be the accumulation and storage of the oils which frequently is phytotoxic. Their position corresponds to an apparent first line of defense at the surface of the plant (Wagner, 1991).

Ivanić and Savin (1976) analyzed the quantity and chemical composition of essential oils from various wild species of Salvia. The results showed that *S. officinalis* has the highest essential oil yield among Salvia species, along with a higher total ketone content and a lower total alcohol content. Bernotienė *et al*. (2007) compared the essential oils of *S. officinalis* of various chemotypes between ISO 9909 and The German Drug Codex. They investigated that ISO 9909 has following constituents in the sage essential oils: *cis*-thujone (18.0–43.0%), camphor (4.5–24.5%), 1,8-cineole (5.5–13.0%), *trans*-thujone (3.0–8.5%), α-humulene (≤12.0%), α-pinene (1.0–6.5%), camphene (1.5–7.0%), limonene (0.5–3.0%), bornyl acetate (≤2.5%) and linalool + linalyl acetate (≤1.0%). While The German Drug Codex requirements differ from the above ISO as following: thujones (≥20.0%), camphor (14.0–37.0%), 1,8-cineole (6.0–16.0%), borneol (≤5.0%) and bornyl acetate (≤5.0%). The Codex regulates the amounts of only five compounds, while ISO 9909 – of eleven constituents.

3.2 Biosynthesis of monoterpene synthases in *Salvia officinalis*

As explained above, monoterpene biosynthesis is located in plastids. Accordingly, the monoterpene synthases representing the key enzymes in monoterpene biosynthesis, are located in the chloroplasts. As these enzymes are nuclear gene products, the encoded preproteins reveal an amino-terminal transit peptide for the import into the plastids, where they are proteolytically processed to the mature forms (Wise *et al*., 1998).

All monoterpene synthases convert the same substrate, i.e. geranyl-pyrophosphate, which is produced by the condensation of IPP and DMAPP (Figure 7) to monoterpenes. However, the reaction products could differ significantly depending on the properties of the enzyme. The three most abundant and important monoterpene synthases in *Salvia officinalis* are the cineole synthase, leading directly to 1,8-cineole, the sabinene synthase responsible for the first step in the formation of α- and ß-thujone, and the bornyldiphosphate synthase, which generates the precursor of camphor (Figure 9; Wise *et al*., 1998; Nowak *et al*., 2010; Selmar and Kleinwachter, 2013).

Unfortunately, up to now, no data on the expression of monoterpenes synthases under drought stress are available. However, the cDNAs encoding of these three monoterpene synthases already had been successfully isolated and functionally expressed in *Escherichia coli* (Wise *et al*., 1998).

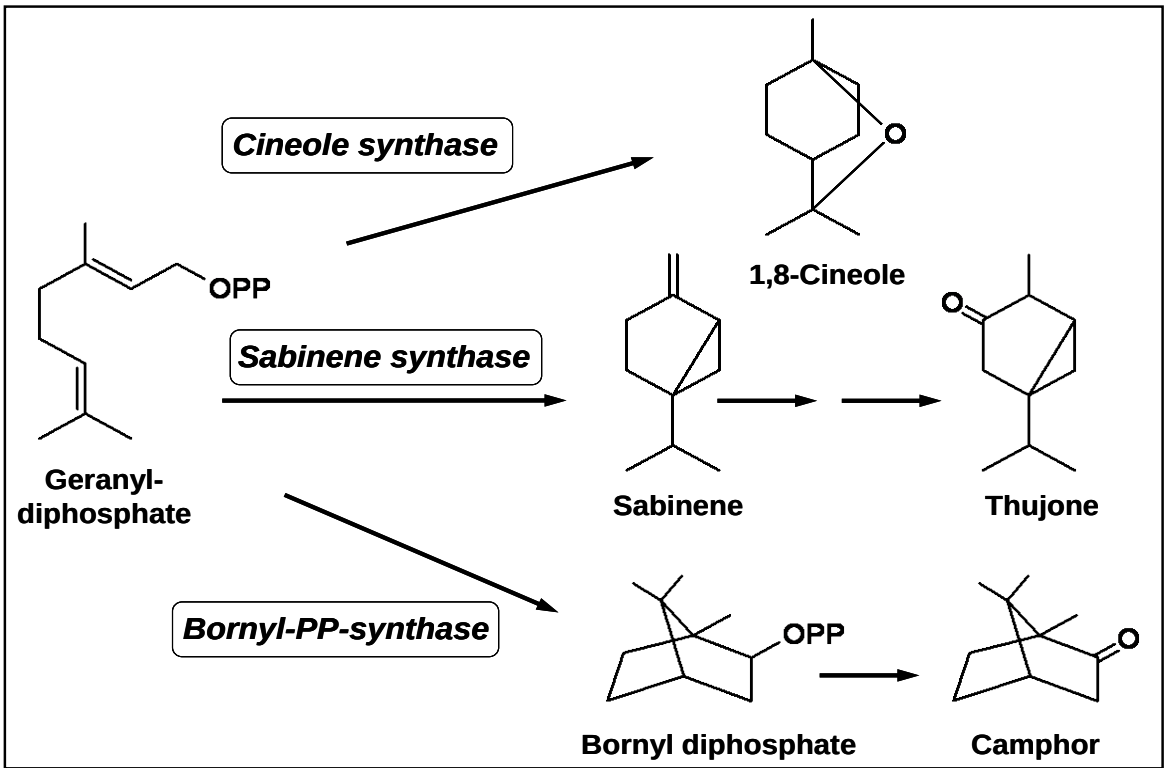


Figure 9: Biosynthesis of the main monoterpenes in sage (> 90% of the total content of *Salvia officinalis*); Selmar and Kleinwächter, 2013).

Grausgruber-Gröger *et al*. (2012) examined the seasonal influence on the three main monoterpenes synthase at the level of mRNA expression in young and still expanding leaves of field-grown sage plants. They found that the transcription of all monoterpene synthase was significantly influenced by cultivar and season. The same research group studied also the impact of exogenously applied plant growth regulators i.e. gibberellic acid and daminozide, on the expression of monoterpene synthases. The authors found that the expression of monoterpene synthase increased with increasing levels of gibberellins, and decreased when gibberellin biosynthesis was blocked with daminozide. On the other hand, after the addition of increasing levels of gibberellins, 1,8-cineole and camphor contents increased, too. However, the

accumulation of α- and ß-thujone was blocked by daminozide (Schmiderer *et al*., 2010). Obviously, the expression of the different monoterpene synthases is regulated differentially.

As stated above, there is no information available on the stress related expression of the monoterpene synthases in plants in general and especially in drought stressed *Salvia officinalis*. Accordingly, this study will contribute to elucidate the complex interactions between drought stress and the synthesis of these enzymes and in particular their regulation, as well as to increase the general understanding of the impact of drought stress on plant secondary metabolism.

III. Materials and Methods

1. Materials

1.1. Plant material

Sage plants (*Salvia officinalis*) were obtained from a local garden centre at Braunschweig, Germany. The growth conditions are described in details in the experimental procedures chapter.

1.2. Reagents and preparation of solutions and buffers

DNA-electrophoresis	Component	Amount
50x TAE (Tris/acetate/ EDTA) DNA electrophoresis buffer	For 1 L Tris Base (MW=121.1) glacial acetic acid 0.5 M EDTA 8.0 (working solution 1x) stored at room temperature	 242 g 57.1 ml 100 ml
SDS-PAGE Gel Solutions		
40% Acrylamide/Bis	For 100 ml acrylamide bis-acrylamide stored at +4 °C	 38.62 g 1.38 g
Stacking gel	For 100 ml 2 M Tris-HCl buffer (pH 6.8) SDS H_2O stored at +4°C	 25 ml 0.4 g 75 ml
Separating gel	For 100 ml 2 M Tris-HCl buffer (pH 8.8) SDS H_2O stored at +4°C	 75 ml 0.4 g 25 ml
5X SDS Loading Sample Buffer	For 100 ml 1 M Tris-HCl buffer (pH 6.8) SDS glycerol	 31.25 ml 10 g 50 ml

	BPB (bromophenol blue) up to 100 ml with d. H_2O stored at room temperature	25 mg
SDS electrophoresis buffer (1x)	For 1 L Tris base glycine 10 % SDS Up to 1000 ml with d. H_2O (No need to adjust pH) stored at +4°C	 3 g 14.4 g 10 ml
10% SDS	For 100 ml SDS up to 1000 ml with d. H_2O heated at 68^{o}C for solubility stored at room temperature	 10 g
10% (w/v) APS	For 1 ml APS up to 1 ml with d. H_2O freshly prepared	 100mg
Staining solution	For 500 ml coomassie-blue R 250 methanol acetic acid up to 500 ml with d. H_2O Filtered before use	 1.2 g 300 ml 60 ml
Destaining solution	For 1 L methanol acetic acid dH_2O	 100 ml 70ml 830 ml
Buffers for affinity purification His6-tagged fusion protein		
½ EQ buffer (25mM Tris HCL, pH 7.5, 500 mM NaCL)	For 100 ml 1 M Tris HCL, pH 7.5 5 M NaCL dH_2O	 2.5 ml 10 ml 87.5 ml

Western Blotting and Immunostaining buffers		
Transfer buffer	For 1 L Glycine Tris base SDS methanol	 2.93g 5.81g 0.375g 200mL
TBS	For 1 L 20 mM Tris/Cl, pH 8.0 150 mM NaCl	 20 ml 30 ml
TBST	For 1 L 50 mM Tris/Cl, pH 8.0 150 mM NaCl 0.1% Tween 20	 20 ml 30 ml 1 ml
Blocking solution	For 100 ml 4% milk powder (non-fat dry milk powder) TBS	 4 g 96 ml
AP buffer	Tris-HCl (pH 9.5) NaCl $MgCl_2$ Tween 20 dH_2O stored at room temperature	2.4228 g 1.16 g 0.204 g 0.1 ml up to 200 ml
BCIP/NBT alkaline phosphatase substrate working solution	AP buffer (pH 9.5) 1% BCIP 1.5% NBT	30 ml 120 µl 120 µl

1.3. Bacterial culture medium

Medium	Ingredients
LB (for 1 Liter) (Luria – Bertani)	To 950 ml of deionized water, the following components were added tryptone 10 g yeast extract 5 g

	NaCl 10 g pH 7.0 was adjusted with 5 N NaOH (take about 0.2 ml). For solid media 15 g of agar were added after adjusting the pH.
SOB (for 1 Liter)	20.0 g Tryptone 5.0 g Yeast Extract 0.5 g NaCl 186.0 mg KCl pH 7.0 was adjusted with 5 N NaOH (approximately 0.2 ml) for solid media, add 15 g of agar after adjusting the pH Adjust the volume to 1000 ml and sterilize by autoclaving. Once autoclaved, add 10 ml of sterile 1 M Mg^{2+} (e.g. 10 ml of sterile 1 M $MgCl_2$ or sterile 1 M $MgSO_4$)
SOC (For 1 Liter)	The same as components as SOB. After autoclaving, the medium was let cool down to about 60°C and 10 ml of 50% glucose was added.

1.4. Solutions and stock solutions for the transformation and selection of E. *coli*

Solutions	Ingredients
Antibiotics	**Ampicillin stock solution (50 mg/ml)** 0.25 g ampicillin sodium salt in 5 ml of deionized water, Filter-sterilize and stored in aliquots at 20°C. **Chloramphenicol stock solution (30 mg/ml)** 0.3 g chloramphenicol in 10 ml of ethanol (96%). Stored in aliquots at 20°C. **Kanamycin (sulphate)** 30 mg/ml in deionized water. Filter- sterilized and stored in aliquots at 20°C.
IPTG solution 100 mM	238.3 mg IPTG in 10 ml of deionized water. Filter-sterilized and stored in aliquots at -20°C

1.5. Bacterial strains (*Escherichia coli strains*)

Escherichia coli	Description/Application	Genotype	Supplier
DH5α	Used to maintain the initial	F^- φ80*lacZ*ΔM15 Δ(*lac*ZYA-	Invitrogene

	cloning of target DNA into pJET1.2/blunt Cloning Vector	*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	
NovaBlue GigaSingles™	Used for protein expression	Not stated in the manual of the kit	Novagen®
BL21(DE3)	Solubility enhancement and rare codon supplemented	F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3)	Novagen®
BLR(DE3)pLys	High-stringency expression	F^- ompT $hsdS_B(rB^- mB^-)$ gal dcm (DE3) pLysS	Novagen®

1.6. Vectors

Vectors	Description	Supplier
pJET1.2/blunt	Cloning vector	fermentas™
pET-30 Xa/LIC Vector	Expression vector with N-terminal His6-tag	Novagen®
pRSET B	Expression vector with N-terminal His6-tag	Invitrogen

1.7. DNA oligonucleotide primers

Unless stated otherwise, all primers were synthesized in high purity salt free quality at biomers.net (Ulm, Germany).

1.7.1. Specific primers of dehydrin gene isolated from *Salvia miltiorrhiza*

Oligo Name	**Oligo Sequence (5‘ – 3‘)**
SmDehy	GCACGAGGCTCAACTTCCCATC
SmDehyR441bp	GGAACCGATGTGTCTACGCACT

1.7.2. Primers used for the extension of dehydrin fragment at 5´ RACE

Oligo Name	Oligo Sequence (5´– 3´)
SMARTer II A oligonucleotid	AAGCAGTGGTATCAACGCAGAGTACGCGGG
5'-RACE CDS primer A	(T) 25V N–3' (N = A, C, G, or T; V = A, G, or C)
Long SAUPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACG CAGAGT
Short SA UPM	ShortSA UPM: 5'–CTAATACGACTCACTATAGGGC
Nested universal primer A	5'–AAGCAGTGGTATCAACGCAGAGT–3'
GSP ,SmDehy161bp R1	5'–ACCTCCTCCTCGCTCGACG–3'

1.7.3. primers for 3´ RACE extension

Oligo Name	Oligo Sequence (5´ – 3´)
3' RACE adapter	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN
3' RACE outer Primer	GCGAGCACAGAATTAATACGACT
3' RACE inner Primer	CGCGGATCCGAATTAATACGACTCACTATAGG
GSP ,SmDehy161bp	GAT CGC CTC CGA GTT CGA GAA GA

1.7.4. pJET1.2 sequencing primers

Oligo Name	Oligo Sequence (5´ – 3´)
pJET1.2 forward sequencing primer	CGACTCACTATAGGGAGAGCGGC
pJET1.2 reverse sequencing primer	AAGAACATCGATTTTCCATGGCAG

1.7.5. Full length of dehydrin gene from 5´ and 3´ends primers

Oligo Name	Oligo Sequence (5´ – 3´)
Full 5´end dehy2	ATGGCCGAAGAAGCGAAATACAGTTAC
Full 3´end dehyR2	ACG AAC CAA ACA GCA AAA AAC GC

1.7.6. Primers sequences of dehydrin and monoterpene synthases for qRT-PCR

Oligo Name	Accession no.	Oligo Sequence (5´ – 3´)
Sodeh2	JF491334.1	(F) AGGAGGAGAAGCACGAGAGC (R) ACGGGAGCCGATGTGTCTAC
(+)-Bornyl diphosphate synthase	AF051900.1	(F) GAAACTACCAACCTGCCCTTT (R) AAATCCGAGAGCCCCAAATA
1,8-Cineole synthase	AF051899.1	(F) GACGACGAACTGGAGGCTAC (R) GCGGTCAAAATGGCAAGATA
(+)-Sabinene synthase	AF051901.1	(F) GCAAGACGATTTTCCACCAG (R) TTGAGTTTGGCGAGTTTGAA

1.7.7. Primers sequences of reference genes for qRT-PCR

Oligo Name	Oligo Sequence (5´ – 3´)
Actin (Cytoskeletal structural protein)	(F) AGGAACCACCGATCCAGACA (R) GGTGCCCTGAGGTCCTGTT
EF1 α (Elongation factor 1 alpha)	(F) AGACCACCAAGTACTACTGCAC (R) CCACCAATCTTGTACACATCC
Ubiquitin (Ubiquitin protein)	(F) GTTGATTTTTGCTGGGAAGC (R) GATCTTGGCCTTCACGTTGT
18S (18S Ribosomal RNA)	F) CTTCGGGATCGGAGTAATGA (R) GCGGAGTCCTAGAAGCAACA
α -Tubulin Cytoskeletal structural protein	(F) AGAACACTGTTGTAAGGCTCAAC (R) GAGCTTTACTGCCTCGAACATGG
G3PHD(glyceraldehyde-3-phosphate dehydrogenase)	(F)CCCTAGCAAAGATGCCCCAATG (R) AGACCCTCCACAATGCCAAACC

1.7.8. Primers used in Novagen system

Oligo Name	**Oligo Sequence (5´ – 3´)**
LIC sense primer	5' GGTATTGAGGGTCGC ATGGCCGAAGAAGCG– insert-specific sequence 3'
LIC antisense primer	5' AGAGGAGAGTTA GAG CC TTAGCATGCACCACC–insert-specific sequence 3'
pET Upstream primer	ATGCGTCCGGCGTAGA
T7 Terminator primer	GCTAGTTATTGCTCAGCGG

1.7.9. Primers for cloning into pRSET_B

Oligo Name	**Oligo Sequence (5´ – 3´)**
SoDHEcoRI	CTTCGAATTCTTAGCATGCACCACC
DehBamH1	ATTGGATCCGATGGCCGAAGAAGCG
pRSET T7 promoter	ATACGACTCA CTATAGGG
pRSET-Reverse T7 Terminator Primer	TAGTTATTGCTCAGCGGTGG

1.8. Antibodies

Dehydrin K-segment antibodies	Primary antibody (from Timothy J. Close, University of California)
Anti-Rabbit IgG	Second antibody, goat anti-rabbit IgG - alkaline Phosphatase conjugated, (Sigma-Aldrich (Steinheim, Germany)

1.9. Online software and database websites

NCBI (National Center for Biotechnology Information) : http://www.ncbi.nlm.nih.gov/

ChromasPro 2.4.1: http://en.bio-soft.net/dna/chromas.html

Integrated DNA Technology: http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/

Reverse Complement: http://www.bioinformatics.org/sms/index.html

ExPASy translate: http://www.expasy.ch/tools/dna.html

ClustalW2: http://www.ebi.ac.uk/Tools/msa/clustalw2/

Show Translation: http://www.bioinformatics.org/sms/index.html

Banklt: http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank

Protein Information Resource : http://pir.georgetown.edu/

Compute pI/Mw: http://web.expasy.org/compute_pi/

Biolabs, NEBcutter V2.0: http://tools.neb.com/NEBcutter2/

Oligo Analysis Tool: http://www.operon.com/tools/oligo-analysis-tool.aspx

Primer3: http://frodo.wi.mit.edu/primer3/

DoubleDigest: http://www.thermoscientificbio.com/webtools/doubledigest/

Phylogeny Analysis: http://www.phylogeny.fr/version2_cgi/phylogeny.cgi

Intrinsic Protein Disorder Prediction 1.5: http://globplot.embl.de

ChloroP: http://www.cbs.dtu.dk/services/ChloroP/

Prediction of protein localization sites in cells: http://psort.hgc.jp/form.html

2. Experimental procedures

2.1. Molecular biology analysis methods

2.1.1. RACE-PCR-based isolation of full length dehydrin gene from *Salvia officinalis*

2.1.1.1. Design of dehydrin gene specific primer

Specific dehydrin gene primers were designed according to the general primer design criteria using internet website Integrated DNA Technology (listed in III.1.9) and the published coding sequence (CDS) of dehydrin gene from *Salvia miltiorrhiza* (accession number: AY695932.1), see (III.1.7.1).

2.1.1.2. Plant material

Sage plants were grown in a greenhouse under well-watered conditions for two weeks. Drought stress was induced by interrupting the water supply of the leaves by detaching them. Middle aged leaves were incubated for 24h and others for 48h, respectively. In addition, the well-watered leaves (control) were frozen in liquid nitrogen directly after detaching.

2.1.1.3. RNA extraction from sage leaves

100 mg of leaf materials were ground in liquid nitrogen. Following the manufacturer's protocol (TriFast, peQlab, Germany), the tissues were homogenized in 1ml of TriFast and incubated for 5 minutes at room temperature. 200µl chloroform were added to the samples. The mixture was incubated for 3 minutes at room temperature. The samples were centrifuged at 12.000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube. The aqueous phase was incubated with 500 µl isopropyl alcohol for 10 minutes at room temperature and then centrifuged at 12000 x g for 10 minutes at 4°C. The RNA pellet was washed with 1 ml 75% ethanol and dried in air. The RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55°C. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. An aliquot of 2 µl of each sample was tested on a 1% agarose gel.

2.1.1.4. Reverse transcription of mRNA (First Strand cDNA synthesis)

To confirm the complete removal of DNA contamination which would negatively interfere with PCR analysis, a DNase digestion step was carried out before transcription. Equal amounts of RNA (1µg) were mixed with 20U Ribonuclease inhibitor, 1U DNase (Fermentas) and 1µl 10X reaction buffer with $MgCl_2$. DEPC-treated water was added to the mixture to the final volume of 10 µl. The mix was incubated for 30 minutes at 37°C. DNase was inactivated by incubation with 1µl 25 mM EDTA at 65°C for 10 minutes. The prepared RNA was used as a template for reverse transcriptase. Reverse transcription reactions were performed according to the manual instructions using the RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) which has lower RNase H activity compared to AMV reverse transcriptase. To improve RT-PCR results, separate step for RNA denaturation and primer annealing was performed: about 1µg RNA mixed with 1µl oligo (dT) 18 were centrifuged briefly and incubated at 65°C for 5 min, chilled on ice, spined down and then the vial was placed on ice. Subsequently, the following components were added:

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 u/=l)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M MuLV Reverse Transcriptase (200 u/=l)	1 µl
Total volume	20 µl

The reaction solution was gently mixed and centrifuged, incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min. The reverse transcription reaction product was directly used in PCR applications.

2.1.1.5. PCR amplification of cDNA (Second strand)

Routine PCR amplification was carried out using the previous cDNA samples and dehydrin gene specific primer as described above (III.1.7.1). About 2 µl of a ten-fold dilution of the cDNA was used as template in PCR amplification. PCR reactions were set up in 25 µl of standerd buffer using Hotstart Taq polymerase (Qiagen, Hilden, Germany) as follows:

Forward Primer (10 pmol)	1.5 µl

Reverse Primer (10 pmol)	1.5 µl
cDNA from RT reaction (1:100 dilution)	2 µl
10X PCR buffer	2.5 µl
10 mM dNTP Mix	0.5 µl
25 mM $MgCl_2$	1.5 µl
DNA polymerase (5 u/=l)	0.25µl
Water, nuclease free	15.25µl
Total volume	25 µl

The cycling conditions were 5 min at 95 °C, 30 cycles (95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min), final extension, 72 °C for 10 min.

2.1.1.6. Agarose gel electrophoresis

For DNA visualization, the samples were mixed with loading buffer (6× loading buffer, Fermentas); agarose gels (1-2.5 % w/v) were used to electrophoretically separate DNA fragments. The agarose concentration was determined according to the expected band size. Agarose was mixed with electrophoresis buffer (1 x TAE) to the desired concentration, and then cooked in a microwave until complete melting. After warming the mixture, ethidium bromide was added to the gel (final concentration 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis.

2.1.1.7. Purification of PCR products for sequencing

The PCR product of approximately 350 to 400 bp was cut out and purified using Fermentase GeneJET, Gel Extraction kit. As described in the manufacturer's protocol with slight modification, equal volume of binding buffer was added to the gel piece. Gel mixture was incubated at 60°C for 10 min. the tube was mixed by inversion every few minutes until the gel piece was completely melted. About 700 µl of the solubilized gel solution was transferred to the GeneJET™ purification column and centrifuged for 1 min at 10.000 rpm. Then the flow-through was discarded and the column was washed by 700 µl washing buffer. Before DNA elution, the GeneJET™ purification column was centrifuged for additional 1 min at 13.000 rpm to remove the remaining ethanol from the purified DNA solution which inhibits downstream enzymatic reactions. 40 µl of Elution Buffer was added to the centre of the purification column membrane.

The column was incubated for 2 min at room temperature and centrifuged for 1 min at 12.000 rpm. The purified DNA was electrophoretically separated on 2 % agrose gel and stained with ethidium bromide before sequencing.

2.1.1.8. DNA sequencing

Nucleotide sequences were determined by the dideoxyribonucleoside chain termination method, which was developed by Frederick Sanger and co-workers in 1977. Primers specific to dehydrin gene were used to read the sequence (listed in III. 1.7.1). The purified PCR product was sequenced automatically by automated DNA sequencing method. The automated DNA sequencing reactions were performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (3100 Genetic Analyzer), Sequence laboratories Göttingen, Germany). The data were obtained as fluorimetric scans from which the sequence was collected using the sequence analysis software. Alignment of DNA sequences was performed using the BLASTX comparison with GenBank data base.

2.1.1.9. Extension of dehydrin gene (SoDHN) by using Rapid Amplification of cDNA Ends (RACE)

In general, Rapid Amplification of cDNA Ends (RACE) technique is used in order to amplify DNA sequences from mRNA template between a defined internal site and unknown sequences at either 5´ or 3´ end of the mRNA. SMART cDNA synthesis (Switching Mechanism At 5´end of RNA Transcript); SMARTer™ RACE kit (Clontech) was used to amplify 5´ and 3´ ends of dehydrin fragment. The key idea of this kit based on SMART Scribe Reverse Transcriptase which is a modified from MMLV RT. This reverse transcriptase exhibits terminal transferase activity, which can reach the end of RNA template and adding 3-5 cytosine residues to the 3´ end of the first strand cDNA. SMART II- primer contains a terminal stretch of guanine (G) residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT, since the template switching activity of the RT occurs only when the enzyme reaches the end of the RNA template (Figure 10).

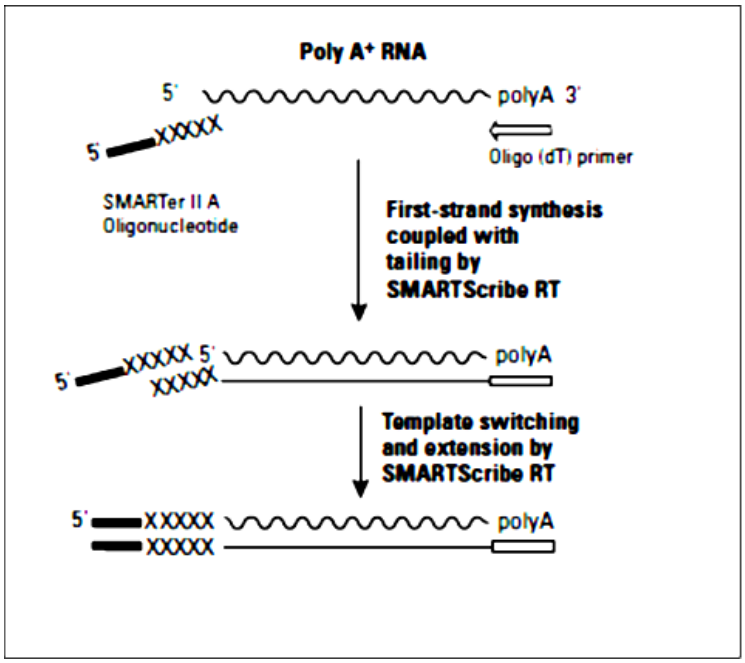


Figure 10: Mechanism of SMARTer cDNA synthesis.

The extension of SoDHN fragment at 5´ RACE

According to the standard protocol described in the manufacturer's instructions. The reaction was carried out in total volume 20 µl using reverse gene-specific primers and kit universal primers (III.1.7.2) as the following procedure:

RNA (200 ng)	6 µl
5'-CDS primer (10 pmol/µl)	1 µl
DTT (0.1M)	2 µl
Nuclease free water	1 µl

RNA was denatured at 70°C for 10 min followed by briefly cooling on ice and the addition of the following reagents:

5x PrimeScriptTM buffer	4 µl
10 mM dNTP mix	1 µl

The reaction was prewarmed at 37°C for 5 min followed by addition of the following components:

PrimeScriptTM Reverse transcriptase (200 U/µl)	0.5 µl
Nuclease free water	4.5 µl

Total volume	20 µl

The reaction mixture was incubated at 42°C for 90 min, followed by heat inactivation of the enzyme at 70°C for 15 min.

The extension of SoDHN fragment at 3´ RACE

The amplification of cDNA ends from 3´ was performed using FirstChoice® RLM-RACE Kit (Ambion) amplification kit. The 3'-RACE cDNA was synthesized using a traditional reverse transcription M-MLV Reverse Transcriptase which lacks terminal transferase activity but with a special 3' RACE Adapter. According to the instructions of the supplier, the following components with 3' RACE adapter primer (III.1.7.3) were mixed gently, spined briefly and incubated at 42°C for one hour.

RNA (200ng)	5 µl
dNTP Mix (2.5 mM)	4 µl
3' RACE Adapter	2 µl
10X RT Buffer	2 µl
RNase Inhibitor (10 U/µL)	1 µl
M-MLV Reverse Transcriptase	1 µl
Nuclease-free Water	5 µl
Total volume	20 µL

2.1.1.10. Polymerase Chain Reaction (PCR) for 5´ RACE and 3´ RACE

PCR amplification for 5´ RACE was performed using annealing gradient in temperature from 54-66 °C. Dehydrin specific reverse primer together with Long SAUPM primers were used followed by two others amplifications using ShortSA UPM and Nested Universal Primer (as listed above in III.1 7.3). PCR reactions were carried in 25 µl of standard buffer using Hotstart Taq polymerase (Qiagen, Hilden, Germany) as follows:

Forward Primer (10 pmol)	2 µl
Reverse Primer (10 pmol)	2 µl
5´ RACE product	0.5 µl

10X PCR buffer	2.5 µl
10 mM dNTP Mix	0.5 µl
25 mM $MgCl_2$	1.5 µl
DNA polymerase (5 u/=l)	0.5 µl
Water, nuclease free	15.5µl
Total volume	25 µl

PCR programme

Step	Temperature, °C	Time	Number of cycles
Initial denaturation /enzyme activation	95 °C	5 min	1
Denaturation	95 °C	30 s	30
Annealing	TM	30 s	
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	1

For 3´ RACE amplification was carried out as described for 5´ RACE (see above) except the exchanging of primers to 3' RACE Outer Primer, 3' RACE Inner Primer and GSP, SmDehyR161bp.

PCR products of 5´ RACE and 3´ RACE electrophoretically were separated on a 2 % agarose gel and stained with ethidium bromide and extracted from the agarose gel as described before.

2.1.1.11. Cloning of 5´ RACE product (partial length) of SoDHN gene

PCR products with 3'-dA overhangs generated by Taq DNA polymerase were used for cloning pJET1.2/blunt cloning vector (Figure 11). The blunt end reaction was set up as follows:

2X reaction buffer	5 µl
PCR product	2 µl
DNA blunted enzyme	0.5 µl
water, nuclease-free	1.5 µl

The mixture was centrifuged for 3-5 s, and incubated at 70°C for 5 min. Then, the reaction was briefly chilled on ice. The ligation reaction was set up by adding the following components:

pJET1.2/blunt cloning vector (50 ng/µl), 0,5 µl T4 DNA ligase (5 u/µl). The mixture was briefly vortexed and centrifuged for 3-5 sec., subsequently, the ligation mixture was incubated at room temperature for 30 min.

The Transformation procedure was performed using Clone JET™ PCR Cloning Kit (Fermentas) which is compatible for all common E.*coli* laboratory strains. Aliquot of 50µl of the DH5α competent cells were thawed on ice. 5 µl of ligation mixture was added to the cells, mixed gently and incubated on ice for 30 minutes. After this, the mixture was heat-shocked for 20 seconds at 42°C in a water bath without shaking, and again incubated for 2 minutes on ice. Then 700 µl of pre-warmed LB medium was added to the mixture. The transformation mixture was incubated at 37°C for 1 hour with shaking at 250 rpm. About 20 µl of transformation mixture was spread on pre-warmed LB-plates containing 50 µg/ml ampicillin. The plates were incubated overnight at 37°C.

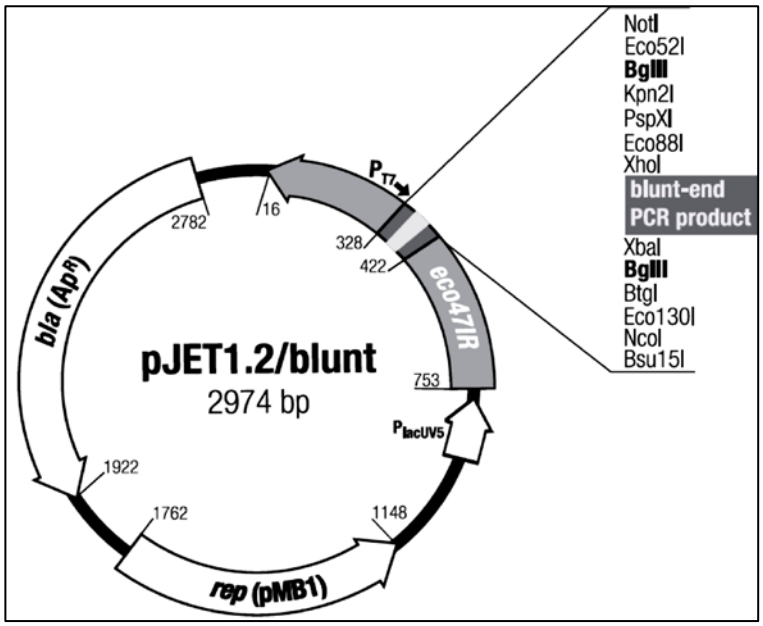


Figure 11: pJET1.2/blunt Vector Map

2.1.1.12. Plasmid Miniprep

Isolation of DNA plasmid was performed using GeneJET™ Plasmid Miniprep Kit (Fermentas). As described in the instructions of the supplier with slight modification, about 5 ml of LB overnight culture containing the appropriate antibiotic was harvested by centrifugation at 12.000 rpm. The pellet resuspended in 250 µl of the Resuspension Solution. After that, 250 µl of the Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous. For neutralization, about 350 µl of the Neutralization Solution was added and immediately mixed thoroughly by inverting the tube 4-6 times. Followed by centrifugation for 5 min at 13.000 rpm to pellet cell debris and chromosomal DNA. Then the supernatant was transferred to a GeneJET™ spin column and centrifuged for 1 min at 12.0000 rpm. The plasmid DNA bound to GeneJET™ spin column was washed twice by 500 µl of the wash solution. After discarding the flow-through, the GeneJET™ spin column was centrifuged for an additional 1 min to remove residual wash solution. Plasmid DNA was eluted by 50 µl of warm elution buffer after incubation for 2 min at room temperature. Followed by centrifugation for 2 min at 12.0000 rpm.

The purified plasmid DNA was sent to sequence using the pJET1.2/blunt cloning vector primers (III.1.7.4).

2.1.1.13. The Amplification of full length of SoDHN gene from 5´ and 3´ends

PCR amplification was carried out using cDNA samples and dehydrin gene specific primers (III.1.7.5). PCR reactions were set up in 50µl of standard buffer (as described in III. 2.1.1.5.). The PCR product of approximately 1000bp and 400bp respectively, were electrophoretically separated on a 1 % agarose gel, cut out and purified. After gel extraction, the two amplified fragments were cloned into pJET vector as described before. The purified PCR products were sent to sequence using the pJET1.2/blunt cloning vector primers (III.1.7.4). The sequencing was repeated three times. The obtained sequences were aligned together to check the precision of sequencing. The submission of the full length dehydrin sequence was performed using the website of NCBI /gene bank.

2.1.2. Quantification of dehdyrin (SoDHN) and monoterpene synthases expression using Real Time PCR

2.1.2.1. Plant material and stress treatment

Sage plants were grown in greenhouse under well-watered conditions for two weeks. Drought stress was applied by detaching the leaves. The leaves were left for 0, 2h, 4h, 6h, 12h and 24h hours on the lab bench at room temperature. Immediately after incubation time, samples were frozen in liquid nitrogen and stored at – 80 C.

2.1.2.2. Total RNA extraction

Technically, the high purity and integrity of RNA are highly recommended for RT-qPCR experimental workflow according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines. Since contaminations in the RNA sample may lead to inhibition of the RT. Consequently inaccurate quantification results will be obtained.

Three commercially available kits were used for RNA extraction: Rneasy plant Mini Kit with DNA-eliminator colums, QIAGEN GmbH), QIA RNeasy plus Minikit (QIAGEN) and RNAse free DNAse I – Set (QIAGEN). The isolation procedure started with the addition of 450µl lysis buffer (RLC buffer, Rneasy plant Mini Kit QIAGEN GmbH), which had been mixed with β-mercaptoethanol (10µl /mL RLC lysis buffer), to 100 mg of the ground material. The suspension was immediately homogenized and incubated for 3 min at 56 °C. After centrifugation at 13.000 rpm for 3 min, 400 µl of the supernatant was applied to a filtration column (QiaShredder spin column, QIAGEN GmbH). Subsequently, the suspension was poured completely onto a QiaShredder spin column. The column was centrifuged for 2 min at 13.000 rpm. The following steps including a first digestion step directly on the RNA binding column were carried out according to the manufacturer's instructions(RNeasy mini column, QIAGEN GmbH). A second DNA digestion step was performed to ensure that the RNA sample free from any genomic DNA. For this second digestion, 10µl Rnasefree DNase I and 70µl buffer RDD were added to the eluted RNA solution and brought up to a final volume of 100µl using RNase-free water. The samples were incubated for 30 min at 28 ºC. To wish the spin column membrane, about 350 µl buffer RW1 was added to the Rneasy spin column and centrifuged at10.000 for 30 sec. Then 500 µl buffer RPE were added and centrifuged for 30 sec at 10.000 rpm. This step was repeated twice. After discard the flow-through, the RNeasy spin column was centrifuged for an

additional 1 min at 10.000 rpm to remove residual wash solution. For RNA elution about 40µl Rneasy free water was added. After 2 min, the column was centrifuged for 1 min at 10.000 rpm. The eluted RNA samples were stored at −80 °C.

2.1.2.3. Determination of RNA quantity and quality

The quantity of the total RNA were determined at wavelength of 260 nm by using an UV– spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies,North Carolina, USA). For RNA quality, RNA samples were mixed with RNA loading buffer (6× RNA loading buffer, Fermentas) and visualized by ethidium bromide after electrophoresis through 2% agarose gels.

2.1.2.4. Reverse transcription (RT)

RNA samples had been standardized to contain the same amount of total RNA (50 ng/µL) before they were used as a template for reverse transcriptase. The standard curve were standardized of mixed all RNA samples (500 ng/µL). Reverse transcription reactions were performed using SuperScript III Reverse Transcriptase reagent kit (Invitrogen). A separate step for RNA denaturation and primer annealing was performed as follows: about 11µl RNA (50 ng/µl) mixed with 1µl oligo (dT) 20 primer and 1µl dNTP mix (10 mM) were centrifuged briefly and incubated at 65°C for 5 min. After chilling on ice, spanning down, the vial back was placed on ice. Then the following components were added:

5X Reaction Buffer	4 µl
RNAseOUT (40U/µl)	1 µl
DDT (0.1 M)	1 µl
SuperScript III Reverse Transcriptase	1 µl
Total volume	20 µl

The mix was gently mixed and centrifuged, then incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min. cDNA samples were diluted 1:10 and stored at -20 °C until being used in PCR applications.

2.1.2.5. Selection of reference genes and primer design

Quantitive real time PCR (qPCR) is the most sensitive method for determining gene expression. The relative quantification of the expression of a target gene is measured with respect to a stably expressed reference gene (housekeeping genes known as internal reference gene). The major challenge for the reliable quantification of gene expression using Real Time PCR is the selection of appropriate housekeeping genes. In order to achieve this goal, six housekeeping genes were tested for the normalization: actin, EF1α, α -tublulin, ubiquitin, 18S and G3PDH. All of housekeeping genes primers were published in (Yang *et al*., 2010), excluding G3PDH primers were designed form *Salvia officinalis* glyceraldehyde-3-phosphate dehydrogenase accession no. FJ858192.1 (listed in III.1.7.7). PCR products of all housekeeping genes were electrophoretically separated on 2 % agarose gel to check the expected size before sequenced in order to determine the sequences of amplification products.

2.1.2.6. Dehdyrin (SoDHN) and monoterpene synthases primer design

The primer design plays a critical role in qPCR efficiency. The primers for dehdyrin and monoterpene synthases (III.1.7.6) were designed using primer 3 website and checked again by Oligo Analysis Tool website.

2.1.2.7. Quantitative real-time (RT-PCR) reaction

Quantitative real-time (RT-PCR) was performed in The LightCycler® 480 Real-Time PCR (Roch). PCR reaction mixtures were composed as the following:

deionised nuclease-free, DEPC-treated water (ROTH)	12.4 µL
Forward Primer (10 µM)	1.25 µl
Reverse Primer (10 µM)	1.25 µl
PCR buffer (10×, without MgCl2, peQlab)	2.5 µl
dNTP mix (10 µl each, peQlab)	0.5 µl
MgCl2 (25 mM, peQlab)	2.5 µl
cDNA from RT reaction (1:10 dilution) or water as negative control	2 µl
	0.1 µl

Hot Taq DNA polymerase (5UμL−1, peQlab)

sybrGreen (4 fold) , syBr Green INucleic Acid gel stain Biozym 10,000 con 2.5 µl

final volume 25 µl

Each sample was run in triplicates. PCR efficiency was determined in each PCR run according to standard curve by measuring serial dilutions of mixed CDNA (1×10^{-1}, 5×10^{-2}, 2×10^{-2}, 10^{-2}, 4×10^{-3}, 2×10^{-3}, 8×10^{-4}, 4×10^{-4}, 1.6×10^{-4}, 8×10^{-5}, 3.2×10^{-5}, 1.6×10^{-5}), according to (Bohle *et al.*, 2007) for each dilution also in triplicate.

PCR program was carried out using the following protocol:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	20 s	45
primer annealing	60 °C	20 s	
Extension	72 °C	30 s	
detection of fluorescence signal	84 ◦C	15 s	
Final Extension	72 °C	2 min	1
Melting curve analysis	95 °C	1 min	5 step/°C
	55	1.5 min	
	60	1 sec	

Data evaluation for Ct values was performed by calculating the second derivative maximum (XSDM) for each individual amplification curve fitted with a four parametriclogistic model (Jungebloud et al., 2007; Tichopad et al., 2003; Vandesompele et al., 2002).

2.1.3. Monitoring the expression of monoterpene synthases and dehydrin (SoDHN) in intact plants

About one hundred sage (*Saliva officinalis*) plants were used for this experiment (Figure 12). Plants were divided. In each case, Four individual plants were grown in rectangle pot containing a mixture of (peatmoss: soil: sand, 1: 1: 1). The growth conditions were 16/8 (day/night), under 300 µE m^{-2} s^{-1}. The experiment started with an acclimatization phase for one week under well-watered conditions. The drought stress was initiated by steadily reducing the soil water of half of the plants. While the other half of plants were cultivated under well-water conditions. The evapotranspiration of the drought stressed plants was between 70 to 80% of that of the control plants. The soil water content was determined using a TDR-probe (P2Z from IMKO, Ettlingen, Germany). It was around 27 to 28% in the well watered control and around 9 to 10 % in the drought stress experiment. The sampling was carried out after the evapotranspiration in the drought stressed plant was adjusted. The samples were taken 3, 10 and 15 days after drought stress induction. For each treatment samples from three independent plants were pooled to ensure reproducibility of the results. Leaves were divided in young and middle-aged ones. RNA extraction, reverse transcription, real time PCR and data evolution were performed as described before in chapter (2.1.2).

Figure 12. Cultivation of *Salvia officinalis* under well-watered and moderate drought stress conditions (70:80% evapotranspiration).

2.1.4. Expression of the SoDHN protein in E. *coli*

2.1.4.1. Cloning of SoDHN gene using Novagen system

SODHN gene was Sub-cloned from the cloning vector pJET1.2/blunt Vector into ligation-independent cloning (LIC) protein expression vector which was developed for the directional cloning of PCR products (Novagen, WI, USA) (Figure 13). According to Xa/LIC strategy (Novagen), the open reading frame (ORF) of SODHN was inserted into the ligation independent cloning (LIC) site using primers (III. 1.7. 8) designed for the LIC cloning system. The first methionine of SODHN starts directly after the tag, and the translation stops by using the stop codon of SODHN.

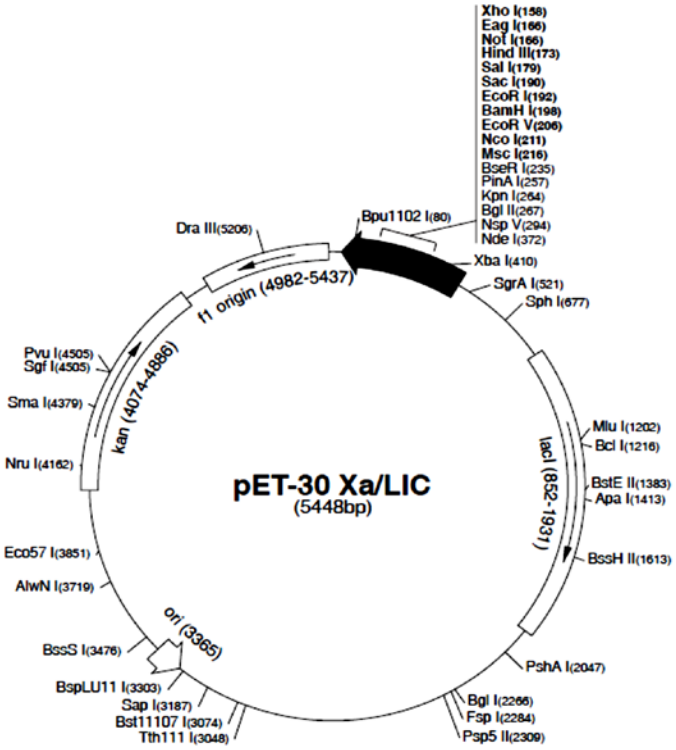


Figure13: pET Xa/LIC Vector

PCR reaction was carried out according to the manufacturer's instructions recommendations (Novagen). KOD Hot Start DNA Polymerase (recombinant from *Thermococcus kodakaraensis*) was used for amplification. The advantage of this enzyme that the enzyme's 3′→5′ exonuclease-dependent proofreading activity results in a lower PCR mutation

frequency than any other commercially available DNA polymerase, in addition to the robust elongation rates. Using the primers LIC (1.7.9), PCR reaction mixtures were composed as the following:

LIC sense primer (10 pmol)	2.0 µl
LIC antisense primer (10 pmol)	2.0 µl
10X PCR buffer	5.0 µl
10 mM dNTP Mix	5.0 µl
25 mM MgCl	3.0 µl
Template DNA	1.0 µl
DNA polymerase (2.5 u/=l)	0.5 µl
Water, nuclease free	31.5µl
Total volume	50 µl

PCR programme

Step	Temperature, °C	Time	Number of cycles
Initial denaturation /enzyme activation	98°C	15 s	1
Denaturation	94°C	10 s	25
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final Extension	72 °C	5 min	1

PCR product was separated on 1 % agarose gel to check the expected size. Subsequently, in order to avoid the generation of false positives which usually generate by residual dNTP, primer dimers and residual polymerase activity, the corresponding band was extracted to prepare the insert for the T4 DNA Polymerase treatment.

2.1.4.2. T4 DNA Polymerase treatment of the target insert

T4 DNA Polymerase treatment was used to generate compatible overhangs on the insert(s) as follows

Purified PCR product	10 µl
10X T4 DNA Polymerase Buffer	2.0 µl

25 mM dATP	2.0 µl
100 mM DTT	1.0 µl
2.5 U/µl T4 DNA Polymerase	0.4 µl
Water, nuclease free	4.6 µl
Total volume	20 µl

The mixture was gently mixed and incubated at 22°C for 30 min. then the reaction was stopped by inactivation the enzyme by incubating at 75°C for 20 min. The prepared insert was stored at –20°C until it was annealed to Xa/LIC vectors.

2.1.4.3. Annealing the Vector and Xa/LIC Insert

The annealing of the vector and the Xa/LIC insert was carried out as the following: Typically, 1.0 µl of the vector was added to 2.0 µl T4 DNA polymerase treated Xa/LIC insert and incubated at 22°C for 5 min. After that 1 µl of 25 mM EDTA was added. The total mixture was gently mixed by stirring with the pipet tip and incubated at 22°C for 5 min.

2.1.4.4. Transformation and heterologous expression of recombinant protein in *E. coli*

The transformation was carried out using the pET-30 E. *coli* expression system. According to the manufacturer's recommendations: Aliquots of 50 µl of three different host cell competent Cells E.*coli* strains (NovaBlue GigaSingles™, BL21(DE3), and BL21(DE3 pLysS (Codon Plus)) were thawed on ice for 2–5 min. 1.0 µl of recombinant plasmid (annealing reaction; 2.1.4.3) was added to each one of the tubes containing host cells. Gently the cells were finger-flicked 1–2 times to mix and returned to the ice. Then, the mixture was heat-shocked for 30 seconds at 42°C in the water bath without shaking, and again incubated for 5 minutes on ice. After that, 250 µl of SOC medium was added to each tube and incubated at 37°C with shaking at 250 rpm for 60 min prior to plating on selective medium. About 25 µl of transformation mixture was spread on pre-warmed SOC-plates containing antibiotics for the plasmid encoded drug resistance (50 µg/ml carbenicillin or ampicillin for the Amp resistance marker, 30 µg/ml kanamycin for the Kan resistance marker, and 34 µg/ml chloramphenicol whena strain carried pLysS. Then, the plates were incubated overnight at 37°C.

2.1.4.5. PCR colony screening

After successful transformation, colony PCR was used as a quickly assess the ability of an individual clone to express a target protein *in vitro* by amplifying a colony with specific LIC primer to check the transformation: A colony approximately about 1.0 mm in diameter was picked from an agar plate using a sterile toothpick followed by touching the toothpick to a plate before transferring the bulk of the colony to the tube. The bacteria were transferred to a 0.5-ml tube containing 50 µl sterile water and vortexed to disperse the cells.

The tube was placed on heated block at 99°C for 5 min to lyse the cells and denature DNases.

To remove cell debris, the heated tubes were spinned at 12,000 × g for 1 min. Approximately, 10 µl of the supernatant was transferred to a fresh 0.5-ml tube for PCR.

Before continuing the protein expression experiment, miniprep was performed as described before for positive colony using appropriate selective antibiotics. After miniprep, a routine PCR amplification was carried using specific pET upstream prime/ T7 terminator Primer. Then, a sample of the purified PCR product was sent directly to automated DNA sequencing using also pET upstream prime/ T7 terminator Primers to check the occurrence of any mutation or frame shift.

2.1.4.6. Heterologous expression of SoDHN protein in E. *coli*

A single colony from a freshly streaked plate was inoculated into 50 ml LB medium containing the appropriate antibiotic and incubated with shaking at 37°C overnight. About 5 ml of the overnight culture was transferred to 100 ml LB medium and incubated at the same growing conditions until OD600 reached to 0.6 to 1.0. An aliquot of 1 ml from the culture was removed for the uninduced control. IPTG was added at a final concentration of 1 mM. The cells continued to grow until 4 hours. 1 ml samples were removed in 1hour intervals for 4 hours of incubation.

2.1.4.7. Extraction of the expressed SoDHN protein from E. *coli* cells

To release the soluble proteins, the BugBuster Protein Extraction Reagent (Novagen) kit was used for the gentle disruption of the cell wall of E. *coli*. As described in the instructions of the supplier with slight modifications: the cells cultures were harvested by centrifugation at 6,500 xg for 5 min. pellet was left to drain out as possible. Then cell pellet was resuspended in 300 µl BugBuster reagent for 1.5 ml culture at room temperature. The mixture was gently vortexed and the foaming was avoided as possible. The cell suspension was incubated on a shaking platform at

a slow setting for 10 min at room temperature. After this, a centrifugation step at 16,000 xg for 20 min at 4°C was done to remove insoluble cell debris. A Further step was performed to analyze the heat stability of the recombinant SoDHN protein by heating the supernatants at 100 °C for 30 min. After cooling to room temperature, the samples were centrifuged at 9,000xg for 15 min at room temperature. The supernatant (soluble extract) before and after heating was transferred to a fresh tube and kept at +20 °C until loaded onto SDS-PAGE.

2.1.4.8. Purification of heterologusly expressed His6-tagged proteins

Protein purification of the SoDHN His6-tagged recombinant protein was carried out using Prepacked HiTrap Chelating column which contains chelating sepharose (GE Healthcare). The purification was performed according to the manufacturer's recommendations as the following: a 1-mL HiTrap Chelating HP column was washed by dH_2O and charged by nickel. Then washed by dH_2O to remove the excess nickel ions followed by equilibration with ½ EQ buffer. About 6 ml of protein soluble extract from heterologusly expressed His6-tagged proteins was applied. The bounded protein was eluted by a stepwise gradient of imidazole (10, 20, 30, 40, 50, 100, 150 and 1000 mM).

2.1.4.9. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

SDS-PAGE was employed to confirm the successful expression of cloned cDNAs in heterologous expression systems. A SDS-PAGE allows separating proteins primarily by size (Laemmli, 1970). The anionic detergent SDS (sodium dodecyl sulphate) binds to proteins, causes penetration of the polypeptides into their primary structure, and serves to give each protein molecule a net negative charge. This charge masks any natural charge of the amino acid residues of the proteins and equalizes the charge along the protein molecules. In addition to reducing agent, such as beta-mercaptoethanol (BME) or dithiothreitol (DTT) disrupts the disulfide bonds which holding protein's tertiary or quaternary structure together. Thus, when an electric current is applied in gel buffer, each protein in the extract migrates toward the positive electrode at a rate inversely proportional to its molecular weight. For highly positively charged proteins, molecular weights calculated from migration during SDS-PAGE may differ significantly from the true molecular weight. Protein samples were mixed with protein loading buffer. After electrophoresis, the gel stained by Coomassie Brilliant Blue for one hour. The protein bands become visible as the blue dye molecules bind to the peptides. In addition to acetic acid in the staining solution fixes

the proteins and dye in place by causing precipitation of the protein by converting it to an insoluble form. Extra dye was removed during the destaining. Electrophoresis gels were prepared using 10 to 12 % acrylamide solutions.

2.1.4.10. Protein sequencing by MALDI-TOF–MS

Matrix assisted laser desorption ionization (MALDI) coupled to time of flight (TOF) was employed for protein identification. In principle, MALDI is a soft ionization technique in which a short laser pulse, instead of continuous laser, of nitrogen gas to ionize molecules. A digested protein with trypsin into short peptides sample is immobilized on metallic Matrix.

Matrix peptides are immobilized on metallic matrix, the mixture of digested protein and matrix crystallizes due to the vacuum environment and then is irradiated with a short laser pulse. The sample molecules and the matrix enter gas phase. This leads to release of matrix, samples molecules, and ions from the target plate. The ions then accelerate in TOF analyzer because they are subject to equal electric field. TOF is a field-free flight tube. The ions travel in a strait and linear direction to the detector.

After electrophoresis, protein bands of interest were excised from the gel by a clean scalpel, washed three times with deionized water and left to dry in speedVac for 30-60 min. The dried protein bands were sent to the AG CPRO, HZI for protein identification by MALDI TOF-MS and MS/MS.

2.1.5. Cloning of SoDHN gene using Invitrogen system

The pRSET B vector (Invitrogen; figure 14) was designed for the expression of polyhistidine-tagged-fusion proteins in *E.coli*. The expression of the gene of interest from pRSETB is controlled by the strong phage T7 promoter.

2.1.5.1. Construction of dehydrin/pRSET B plasmid

The construction of the dehydrin/pRSET B plasmid was started by adding the recognition sequence sites of the restriction enzymes Bam H1 and EcoR1 on the dehydrin sequence table (III.1.7.9). The amplification of SoDHN for sub- cloning was performed by PCR using the primer mentioned above. The PCR product was cloned in Clone JET™ PCR Cloning Kit as described before in section (III. 2.1.1.11). The double digestion was carried out for dehydrin gene and pRSET B plasmid as the following:

DNA template (up to 1 µg)	10 µl
Buffer 2x Tango	2.0 µl
2-fold excess of BamH1	0.5 µl
EcoRI	0.25 µl
Nuclease free water, up to	20 µl

The mixture was incubated at 37°C for 2 hours. After incubation the digestion efficiency was checked by gel electrophoresis then followed by gel extraction. The removal of the terminal 5-phosphate groups was carried out to suppress self-ligation and circularization of the plasmid DNA. A dephosphorylation step was carried out using Shrimp Alkaline Phosphatase (Fermentas). The dephosphorylation was carried out as the following:

Linear Plasmid DNA (1 µg)	4 µl
10X Buffer	2 µl
Alkaline Phosphatase	1µl
Water, nuclease-free, up to	20µl

The reaction was mixed thoroughly, spinned briefly down and incubated for 30 min at 37°C. The reaction was stopped by heating for 5 min at 75°C followed by purification.

After successful digestion and dephosphorylation, the digested dehydrin DNA sequence was inserted in pRSET B plasmid. The dehydrin DNA sequence includes an ATG translation initiation codon and stop codon. The restriction sites were introduced into the sequence to positioned dehydrin DNA sequence downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, and a polyhistidine tag that permits the use of affinity chromatography for the purification of fusion protein. Standard ligation reaction was performed as the following:

Digested vector 50 ng	1 µl
Digested insert 50 ng	1 µl
5x ligation buffer	4 µl
T4-DNA-Ligase (5U/ µl)	1 µl

Nuclease free water up to 20 µl

The constructed plasmid was sequenced to ensure the presence of the desired insert and no frame shifts were detected.

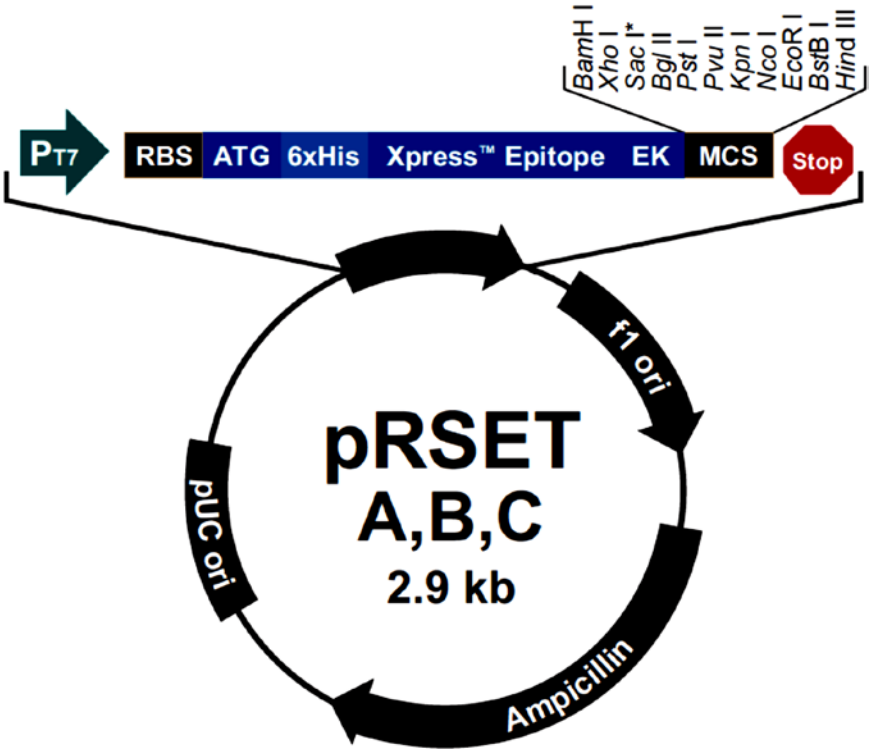


Figure 14: pRSET B vector

2.1.6. Protein extraction

Protein was extracted from plant material using Trifast kit (peQlab). According to the manufacturer's recommendations:the proteins were extracted from the ethanol/phenol supernatant with 1.5 ml isopropanol. The samples were incubated at room temperature for 10 minutes. The extracts were centrifuged at 12,000 x g for 10 minutes at 4 °C. After removing the supernatant, the protein pellet was washed 3 times with 2 ml solution of 0.3 M guanidinium hydrochloride in 95 % ethanol. The samples were kept in the washing solution for 20 minutes at room temperature before were centrifuged at 7,500 x g for 5 minutes at 4 °C. The protein pellet was washed once with 2 ml of 100 % ethanol and stored for 20 minutes at room temperature. Then centrifuged at 7,500 x g for 5 minutes at 4°C. Subsequently, the protein pellet was dried from residual ethanol in suction hood for 30 to 60 min. Protein solubilization was carried out using 50 µl 1 % SDS.

2.1.7. Determination of the protein concentration

Plant proteins concentrations were determined according to the Bradford kit (Roth) (Bradford, 1976). In principle, the stain coomassie Brilliant Blue-G250 binds primarily to basic amino acids. The binding causes a shift in the absorbance from 465 nm to an intense band at 595 nm. This absorption change is proportional to the protein concentration over a wide range. The absorption was measure in a photometric cuvette of one cm width at 595 nm in an UV/VIS spectrophotometer. Protein concentrations were calculated from a calibration curve, which was prepared for each determinations using 1 to 10 µg /ml bovine serum albumin (BSA) as standard. For protein determination, about a 20µL of protein extraction was added to 100µL of 10% TCA and kept on ice for 10 min. Protein was precipitated by centrifugation at 4°C for 5 min at maximal speed. After removing the supernatant, 100 µL of 0.1N NaOH was added to the pellet. An aliquot of 20µL was added to 1 mL of Bradford reagent, mixed well, after 5 min the absorbance was measured at 595nm. The protein concentration in the sample was calculated using the standard curve. About 20µg protein was loaded per lane in the protein SDS-PAGE.

2.1.8. Western Blotting and Immunostaining

After protein electrophoretic separation, proteins were transferred to nitrocellulose membrane (using Mini Trans-Blot Cell, Bio-Rad). The nitrocellulose membrane was washed for 5 minutes in distilled water on a shaker (in a small plastic container). The membrane was soaked in 15mL TBS for 10 min. Subsequently, The membrane were blocked with 5% milk in 1x TBST for 1 hour at room temperature to block any non-specific binding sites and to eliminate the background due to the unspecific binding of the antibodies to the membrane. Then, the membrane was incubated for 60 min in TBS + 4% milk powder with an aliquot of the first antiserum 1:1000 dilution. The membrane was washed 3 times each 10 minutes in TBST. After washing, the membrane was incubated for 60 min in TBS+ 4% milk powder including an aliquot of the second antibody (conjugated with alkaline Phosphatase). The second antibody was usually diluted 1:10000. Then the membrane was washed 5times each 5 minutes in TBST. For detection, BCIP/NBT alkaline phosphatase substrate solution was used. The membrane was incubated in 30 ml AP buffer for 30 min in the dark at room temperature until the appearance of the signal.

2.2. Quantification of monoterpenes using GC

100 mg of frozen powder of leaves was mixed with 1 mL hexane and teteradecan as internal standard (20 µl tetradecan in 100 ml hexane) in a closed reaction vial 2 ml. Each sample was analyzed twice as independent replicates. The mixture was incubated in an ultrasonic bath for one hour at 15 °C. After incubation, the mixture was centrifuged (10 min at 13200 x g). The hexane phase was filtrated (0.45 µm) and used for gas chromatography on a DB1-like Zebron ZB-AAA capillary column (10 m, 0.25 mm). Injection volume was 1,5 µL, split ratio1:100, flow rate 450 mL He / min. Separation of the different compounds was achieved by using the following Temperature program: 2 min for 60 °C, followed by a 15°C / min-gradient up to 180 °C. Then a 20 °C / min-gradient up to 220 °C, followed which was held for 3 min. Detection was performed using a flame ionization detector. Quantification was performed in relation to area under the peak was compared to that of authentic standards (campher, thujone and cineole, Carl Roth GmbH, Karlsruhe). The standard was carried out by using tetradecan , campher, thujone and cineole in diluted in hexane. The dilution factor of thujone cineole and camphor was 1:2500, 1:2500 and 1:2358, respectively. The retention time was 3.32, 4.68, 5.6 and 6,48 for cineole, α- β-thujone, camphor and tetradecan, respectively (see appentix).

2.3. Statistical analysis

The data of qRT-PCR represent represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments. The data points of monoterpenes quantification represent the mean of 2 independent experiments. Error bars represent ± standard deviation.

IV. Results and Discussion

This investigation is aimed to contribute in elucidating the complex metabolic situation of plant responses to drought stress impacting on secondary metabolism. Accordingly, a reliable molecular stress marker is required. In higher plants, dehydrins frequently are synthesized in response to various environmental stresses (i.e., water deficit, high and low temperatures, salinity etc.; Hundertmark and Hincha, 2008; Ingram and Bartels, 1996; Bray, 2004; Wahid and Close, 2007). As the major elicitor for dehydrin synthesis generally is drought stress, dehydrin expression is used as corresponding marker for drought stress. Dehydrins are categorized as group II of highly hydrophilic proteins known as LEA (Late Embryogenesis Abundant) or RAB proteins (Responsive to ABA) and are considered as appropriate stress marker. Thus, the major goal of this investigation is to develop a reliable system to quantify the expression of dehydrins in sage in response to drought stress. Up to the beginning of this study, there was no dehydrins isolated from *Salvia officinalis.* Consequently, for a better understanding, the isolation and characterization as well as the study of the behavior expression profile of dehydrin gene from *Salvia officinalis* is a prerequisite.

The partial length of dehydrin from *Salvia officinalis* will be detected by using primers designed on the basis of a known sequence of a dehydrin from *Salvia miltiorrhiza.* In order to gain the full length of dehydrin from *Salvia officinalis,* the Rapid Amplification of cDNA Ends (RACE) technique (see III. 2.1.1.9) is used to elongate the corresponding PCR product from cDNA template between a defined internal site and unknown sequences at either 5´ or 3´ end of the mRNA. Afterwards, the gene has to be characterized before it could be utilized as reliable stress marker for the investigation of the impact of drought stress on the synthesis of monoterpenes in *Salvia officinalis.*

Due to the large range of different aspects and methods used to elucidate the questions mentioned, it is appropriate to discuss the corresponding topics, directly after the specific results have been presented.

4.1. Isolation and characterization of a dehydrin gene from *Salvia officinalis* as putative stress marker

4.1.1. PCR amplification of partial length of dehydrin gene

In order to get access to dehydrins from *Salvia officinalis*, RNA was extracted from sage leaves and PCR amplification was performed using the forward and reverse gene specific primer pairs, designed on the knowledge of a dehydrin from *Salvia miltiorrhiza* (III. 2.7.1). In order to facilitate the verification, corresponding samples were taken from control and stressed leaves. The quality of the successfully extracted RNAs is documented in Figure 15.

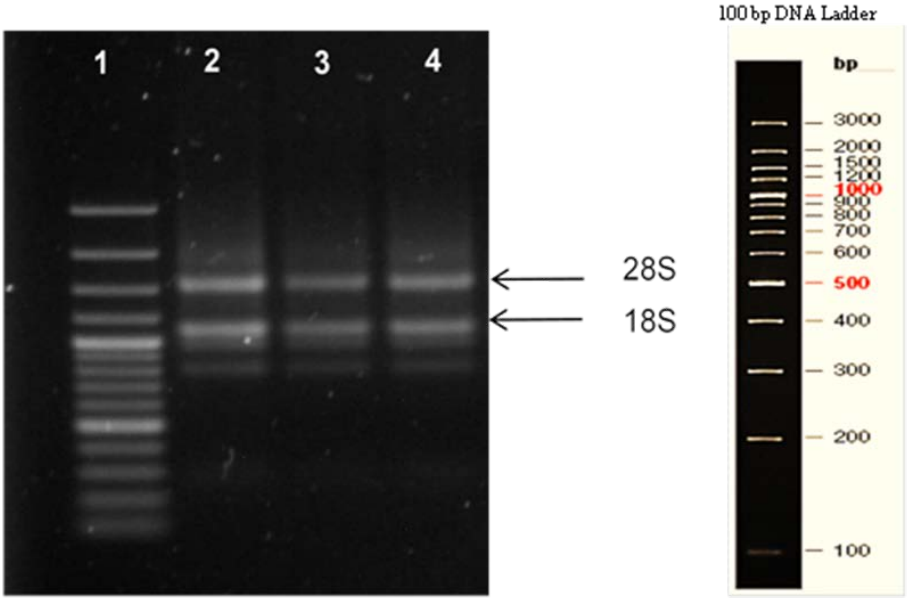


Figure 15: Gel electrophoresis of total RNA extracted from sage leaves
lane 1: marker, lane 2: control, lane 3: stressed 24h, lane 4: stressed 48h
(drought stress was induced by detaching the leaves and blocking the water supply)

These RNAs had been transcribed into cDNAs using oligo (dT) primers. The first strand cDNA was used as a template for the subsequence PCR (see III.2.1.1.4) employing the *S. miltiorrhiza* primes. As result, one PCR product was detectable. It had a length of 350 bp (Figure 16) revealing the expected size of partial length of dehydrin according to the primers design. The sequence was determined. It has similarity to other plant dehydrins. Yet, it is interesting to state that the putative dehydrin is not only present in the stressed but also in the control leaves. The

dehydrin is constitutively expressed; however, as the assays are not quantitatively, expression strength could not be calculated. Many studies demonstrated that dehydrins show a basic constitutive expression, and their expression strongly is increased under drought stress (Bae *et al*., 2009; Du *et al*., 2011; Hara *et al*., 2011; Pual and Kumar 2013; Xu *et al*., 2008).

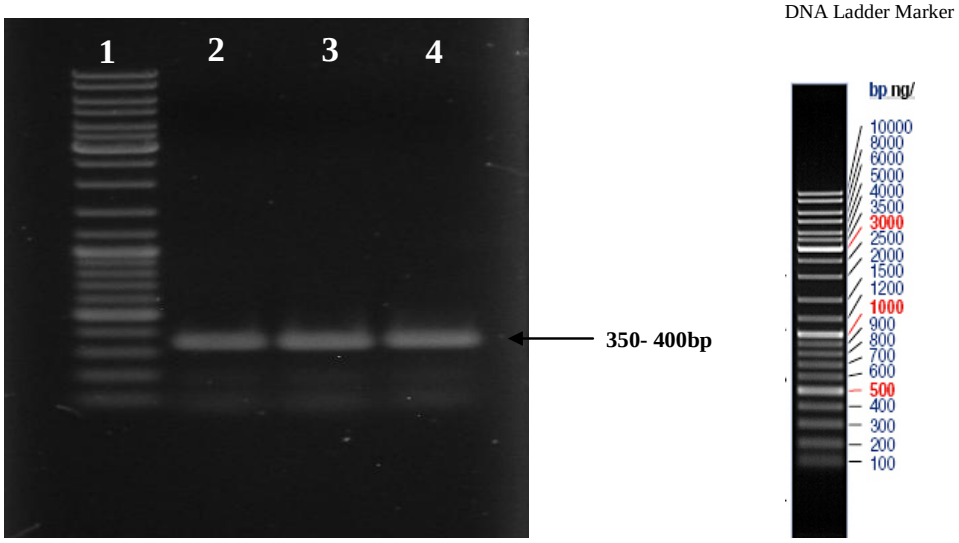


Figure16: Gel electrophoresis for PCR amplification
lane 1: marker, lane 2: control, lane 3: stressed 24h, lane 4: stressed 48h
(drought stress was induced by detaching the leaves and blocking the water supply)

4.1.2. RACE-PCR-based isolation of the full length dehydrin gene

For further characterization of the dehydrin, the full length gene is required. For this, the 274 bp-product was used as template for the RACE techniques.

4.1.2.1. Extension of the dehydrin fragment from 5´and 3´ ends

The extention of cDNA end from 5´ and 3´ was carried out using SAMRT RACE technology that offers a powerful mechanism for generating full-length cDNAs in reverse transcription reactions through the advantage of SMARTScribe Reverse Transcriptase which has terminal transferase activity and can reach to the end of an RNA template (Zhu *et al*., 2001). PCR amplification revealed four products at 5´ and four products at 3´ end. The molecular weight of these products were 450 bp, 550 bp, 700 bp, 1100 bp at the 5´ end and 550 bp, 500 bp, 450 bp, 400 bp at the 3´end (figure 17 A and B respectively).

5´-RACE **3´-RACE**

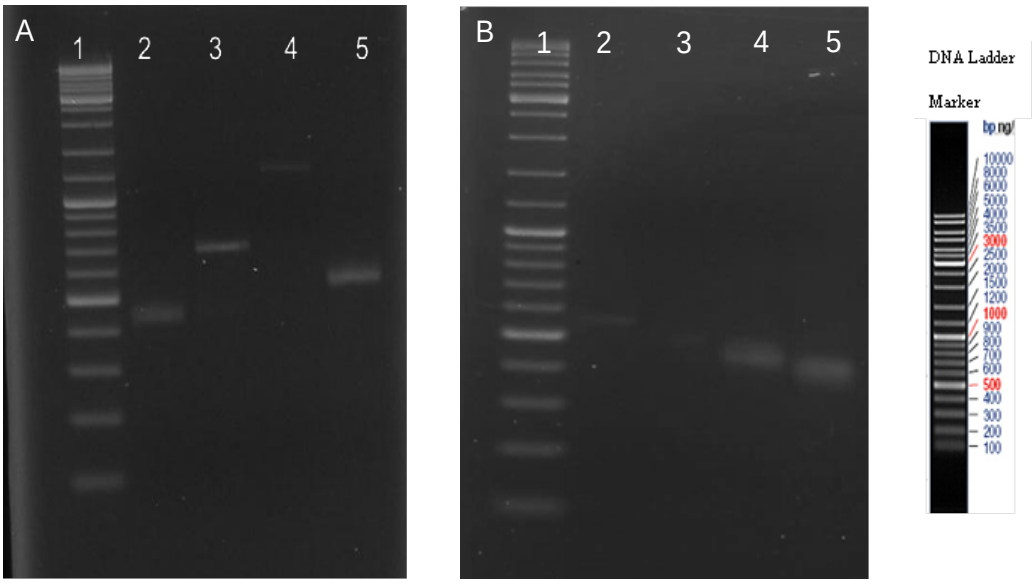


Figure 17: A: PCR amplification products at 5` ends after gel clean (lane 1: marker, 2: 450bp, 3: 700bp, 4:1100bp, 5: 550bp)
B: PCR amplification products at 3` ends after gel clean (lane 1: marker, lane 2: 550bp, 3: 500bp, 4: 450bp,5:400bp)

The both fragments of the highest molecular weight (1100 bp for the 5´ end and 550 bp for the 3´end had been sequenced (Figure 18 A&B) :

3´race fragment :

A

```
    1 A  P  P  P  P  P  A  P  V  A  V  D  H  G  P  I  T  P  E  A
    1 GCGCCGCCGCCGCCGCCTGCGCCGGTGGCCGTCGATCACGGCCCCATCACTCCGGAGGCG
   21 E  G  K  E  K  K  G  F  L  D  K  I  K  E  K  L  P  G  Y  H
   61 GAGGGAAAGGAAAAGAAGGGTTTCTTGGATAAGATCAAGGAGAAACTGCCTGGCTACCAC
   41 P  K  S  D  E  E  K  E  K  E  K  E  K  E  G  G  A  C  *  V
  121 CCCAAGAGTGATGAGGAGAAGGAAAAGGAGAAGGAAAAGGAGGGTGGTGCATGCTAAGTT
   61 N  N  Q  V  K  W  G  L  L  I  I  F  C  D  F  Q  V  L  F  I
  181 AATAATCAAGTCAAGTGGGGTTTGTTGATTATATTTTGTGATTTTCAAGTGTTATTTATT
   81 D  A  F  P  M  F  P  I  F  P  L  L  L  L  C  *  L  S  C  I
  241 GATGCTTTTCCAATGTTTCCTATTTTCCCACTTTTGCTACTTTGTTAATTAAGTTGTATT
  101 W  G  F  H  E  L  H  M  C  M  C  S  L  L  *  K  K  K  T  Y
  301 TGGGGTTTTCATGAATTACATATGTGTATGTGCAGTCTTTTGTAAAAAAAAAAAACCTAT
  121 S  E  S  Y  *  F  G  S  A  I  F  L  E  D  L  L  Q  Y  S  Q
  361 AGTGAGTCGTATTAATTCGGATCCGCGATCTTTCTAGAAGATCTCCTACAATATTCTCAG
  141 L  P  W  K  I  D  V  L  L  L  F  S  Q  D  F  Q  A  V  Y  *
  421 CTGCCATGGAAAATCGATGTTCTTCTTTTATTCTCTCAAGATTTTCAGGCTGTATATTAA
  161 N  L  Y  *  E
  481 AACTTATATTAAGAAC
```

5´race fragment :

B

```
   1 H  L  C  L  N  T  I  S  I  R  R  N  T  T  H  Y  R  E  S  G
   1 CACTTGTGCCTGAACACCATATCCATCCGGCGTAATACGACTCACTATAGGGAGAGCGGC
  21 R  Q  I  F  R  M  A  R  V  F  Q  Q  D  L  I  R  L  T  I  G
  61 CGCCAGATCTTCCGGATGGCTCGAGTTTTTCAGCAAGATCTAATACGACTCACTATAGGG
  41 Q  A  V  V  S  T  Q  S  T  R  G  P  I  Q  I  Q  S  Q  L  P
 121 CAAGCAGTGGTATCAACGCAGAGTACGCGGGGACCCATTCAAATTCAATCTCAACTTCCA
  61 F  L  Q  L  I  S  F  F  K  M  A  E  E  A  K  Y  S  Y  H  E
 181 TTTCTACAACTCATCAGTTTTTTCAAAATGGCCGAAGAAGCGAAATACAGTTACCACGAG
  81 T  T  K  P  A  A  E  E  P  C  A  P  A  V  E  V  E  S  T  D
 241 ACCACCAAGCCCGCCGCCGAGGAGCCGTGCGCCCCCGCCGTTGAAGTCGAGAGCACCGAT
 101 R  G  L  F  G  F  G  K  K  K  E  E  E  K  C  E  E  T  T  I
 301 CGCGGCCTCTTTGGCTTCGGGAAGAAGAAGGAGGAGGAGAAGTGCGAGGAGACGACGATC
 121 A  S  E  F  E  K  K  V  Q  V  C  E  A  E  E  K  K  E  H  H
 361 GCCTCCGAGTTCGAGAAGAAGGTGCAGGTCTGCGAGGCGGAGGAGAAGAAGGAGCATCAT
 141 A  P  V  T  V  P  A  P  V  P  L  P  V  K  E  E  E  K  H  E
 421 GCGCCGGTGACGGTGCCGGCGCCGGTGCCGCTGCCGGTGAAGGAGGAGGAGAAGCACGAG
 161 S  L  I  E  K  L  R  R  S  D  S  S  S  S  S  S  S  E  E  E
 481 AGCTTGATCGAGAAGCTTCGCAGATCCGATAGCTCCAGCAGTTCGTCGAGCGAGGAGGAG
 181 V  E  G  E  N  G  E  K  I  K  R  K  K  K  K  G  L  K  E  K
 541 GTAGAGGGGGAAAACGGGGAGAAGATTAAGAGGAAGAAGAAGAAAGGTTTGAAGGAGAAG
 201 I  L  G  D  K  K  E  E  E  K  K  C  V  D  T  S  V  P  S  F
 601 ATCTTGGGAGATAAGAAAGAAGAAGAGAAGAAGTGCGTAGACACATCGGTTCCATCTTTC
 221 *  K  I  S  Y  N
 661 TAGAAGATCTCCTACAATT
```

Figure 18 Sequence analysis. A: 3´race fragment : Stop codon: TAA; yellow highlighted: coding region; a 3' poly(A) tract were identified; grey highlighted: 3´ UTR untranslated region (177 nucleotides = 59 amino acids).
B: 5´race fragment : Start codon: ATG; yellow highlighted: coding region; grey highlighted: oligonucleotide adapter primer + 5´ UTR (untranslated region, 207 nucleotides = 69 amino acids).

4.1.2.2. Polymerase Chain Reaction (PCR) and cloning the full length dehydrin gene

The sequences had been analyzed based on the known primer sequences from SAMRT RACE cDNA Amplification kit (Clontech). Using primers based on the sequences beginning at the start codon (ATG) and ending with the stop codon (TAA), respectively. A further PCR was performed in order to obtain the full length dehydrin gene.

Surprisingly, this PCR revealed two products, one of approximately 1000 bp and one of about 400 bp (Figure 19 A). Both fragments were sent to sequencing using the same primers. The check of the obtained sequences by alignment with the known nucleotide sequences revealed high similarity to plant dehydrin nucleotide sequence. The PCR products of both candidates had been cloned into pJET1.2/blunt vector (Figure 19: B). After cloning, standard PCR was carried out

using pJET1.2 sequencing primers (III.1.7.4) followed by sequencing (twice) using also pJET1.2 sequencing primers.

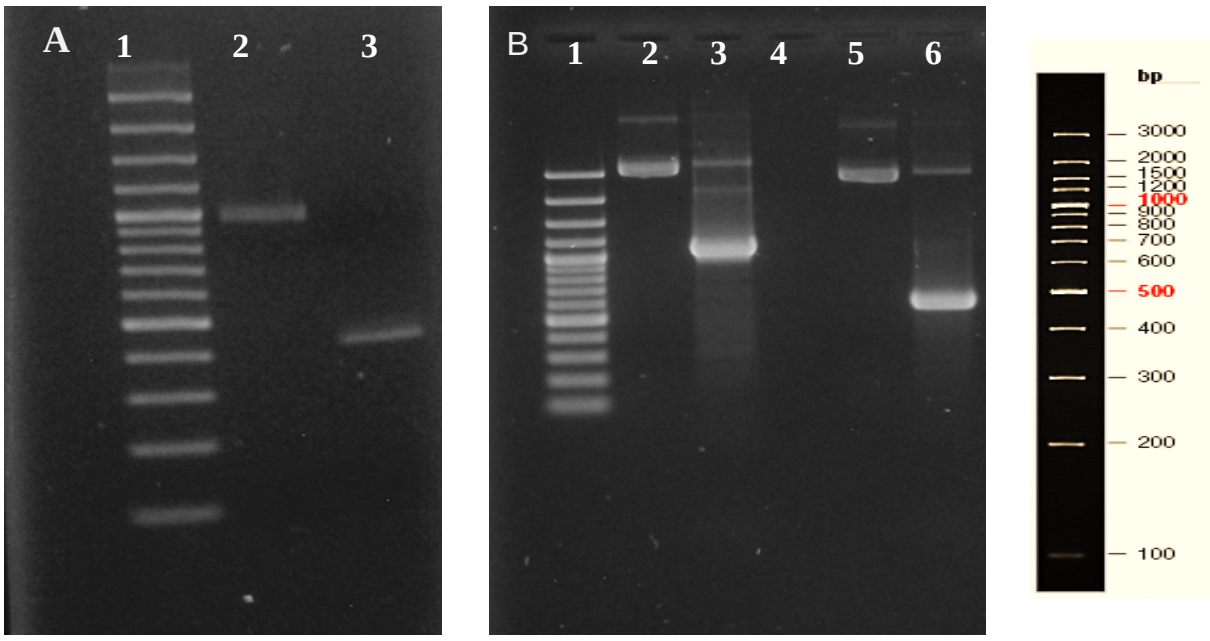


Figure 19 A: agarose gel electrophoresis of the two PCR products after gel clean (lane 1: marker, lane 2: 1000 bp product, lane 3: 400 bp product.

B: agarose gel electrophoresis of the cloned PCR procuts (lane 1: marker, lane 2: miniprep of the vector with the 1000 bp dehydrin product; lane 3: PCR product of miniprep dehydrin 1000bp fragment; lane 4: empty, lane 5: miniprep of the vector of the 400 bp dehydrin products, 5: PCR product of miniprep dehydrin 400bp fragment.

4.1.3. Analysis of sequenced fragments

4.1.3.1 Sequence analysis of the approximately 400 bp fragment

The sequencing of the approximately "400 bp product" (Figure 20) revealed that the sequence exhibits a high similarity to known dehydrin genes at DNA level (i.e., about 89% identity with *Coffea canephora* dehydrin (DH3) mRNA, complete cds; 86% *Eucalyptus globulus* dehydrin 2 (DHN2) gene, complete cds and 81% with *Salvia miltiorrhiza* dehydration protein (bdn1) mRNA. However, it revealed no similarity at amino acid sequence level with other known plant dehydrin protein sequences. Moreover, the approximately 400 bp fragment does not share typical features of plant dehydrins like as K-, S-, Y- segment. Such proteins could be characterized as alternative splicing variants from dehydrin.

```
1   atggccgaagaagcgaaatacggttaccacgagaccaccaagcccgccgccgaggagtcg
     M  A  E  E  A  K  Y  G  Y  H  E  T  T  K  P  A  A  E  E  S
61  tcgatcacggccccatcactccggaggcggagggaaaggaaaagaagggtttcttggata
     S  I  T  A  P  S  L  R  R  R  R  E  R  K  R  R  V  S  W  I
121 agatcaaggagaaactgcctggctaccaccccaagagtgatgaggagaaggaaaaggaga
     R  S  R  R  N  C  L  A  T  T  P  R  V  M  R  R  R  K  R  R
181 aggaaagggagggtggtgcatgctaagttaataatcaagtcaagtggggtttgttgatta
     R  K  G  R  V  V  H  A  K  L  I  I  K  S  S  G  V  C  -  L
241 tattttgtgattttcaagtgttatttgttgatgcttttccaatgtttcctattttcccac
     Y  F  V  I  F  K  C  Y  L  L  M  L  F  Q  C  F  L  F  S  H
301 ttttgctactttgttaattaagttgtatttggggttttcatgaattacatatgtgtatgt
     F  C  Y  F  V  N  -  V  V  F  G  V  F  M  N  Y  I  C  V  C
361 gcagtcttttgtgtacgaaaaaaaaa
     A  V  F  C  V  R  K  K  K
```

Figure 20: DNA and deduced amino acid sequence of dehydrin approximately 400 bp fragment (dehydrin like protein). Yellow highlighted nucleotides are the start and stop codon.

4.1.3.2. Sequence analysis of the 1000 bp fragment (SoDNH gene)

Bioinformatics analysis of SoDNH sequence revealed that the cDNA had a total length of 1000 bp. The putative open reading frame is 735 bp. The ATG (start codon) was found after 207 nucleotides (69 amino acid) from oligonucleotide adapter primer. As well as about 177 nucleotides (59 amino acids) at 3´ UTR (untranslated region) after stop codon (TAA), also a 3' poly (A) tract was identified (Figure 18 A&B).

The amino acid sequence of the *Salvia officinalis* dehydrin (Figure 21) exhibits two K-segments (highly conserved lysine-rich motifs of 15 amino acids) and one S-segment (numerous successive serine residues). The both of K-segments occur near the C-terminus. The first K-segment starts after163 amino acids from start codon, the second K-segment occurs after 209 amino acids from start codon. The S-segment of about 8 serine residues present after 101 amino acids from start codon. S-segment is followed by three glutamic acid residues (SSSSSSSEEE) corresponding to a putative substrate consensus sequence sit for protein kinase2 (CK2) phosphorylation. Furthermore, SoDHN possesses a lysine motif consisting of 6 residues of KRKKKK which is speculated to function as a nuclear targeting signal sequence (NTS). With respect to the typical characterization of plant dehydrins, SoDHN belongs to SK_2 type dehydrins, which generally is up-regulated by low temperature or could be up-regulated by other abiotic stresses such as drought stress.

Figure 21: DNA and predicted amino acid sequence of dehydrin open reading frame (the amino acid sequence of the *Salvia officinalis* dehydrin exhibits two K-segments (dash boxed) and one S-segment (square dot boxed) followed by a putative consensus s substrate sequence site for protein kinase2 (CK2) phosphorylation and nuclear localization signal (solid boxed).

4.1.3.3. SoDNH protein sequence homology analysis

Sequence homology analysis was carried out against nucleotide and protein database of GenBank using BLAST, NCBI tools. SoDNH DNA nucleotide sequence was successfully submitted in gene bank under the accession number: AEB77936.1 using Banklt NCBI website (see III.1. 9). The search in the gene bank database at protein level revealed that the deduced SoDHN amino acid sequence shares around 68% identity to a dehydrin protein from *Salvia miltiorrhiza,* about 56 % identity to a dehydrin protein from *Coffea canephora* , 56% identity to a dehydrin from *Solanum peruvianum,* 53% to the Phosphoprotein ECPP44 from *Daucus carota,* about 40% identity to the dehydrin ERD10 from *Arabidopsis thaliana*.

Using a multiple sequence alignment web site (ClustalW2, see III.1.9), the multi-alignment of the sage dehydrin 's deduced protein (AEB77936.1: dehydrin (*Salvia officinalis*); with those other selected reported dehydrins plant proteins in database such as AAU29458.1: dehydrin (*Salvia miltiorrhiza*), ABC68275.1: dehydrin (*Coffea canephora*), CAH59415.1: dehydrin 1 (*Plantago major*), ADQ73953.1: dehydrin (*Solanum chilense*), ADQ73981.1: dehydrin (*Solanum peruvianum*), AAN78125.1: dehydrin (*Citrus x paradise*) and CAA62449.1 dehydrin (*Arabidopsis thaliana*); (Figure 22). The alignment indicated that SoDNH shares the typical conserved sequence motifs K-segment (EKKGIMDKIKEKLPG) which occurs near the C-terminus. In addition to it contains S-segment LHRSGSSSSSSSEDD (Close, 1996). However, it lacks the Y-segment (T/VDEYGNP) which normally presents at the N-terminus. Nevertheless, this finding is in accordance with Shekhawat *et al*. (2011) who isolated a SK_3-type dehydrin from banana and Rorat *et al*. (2004) who isolated KS-type dehydrins from Solanum species as well as Hara *et al*. (2011) who isolated also a KS-type dehydrin from *Arabidopsis thaliana.*

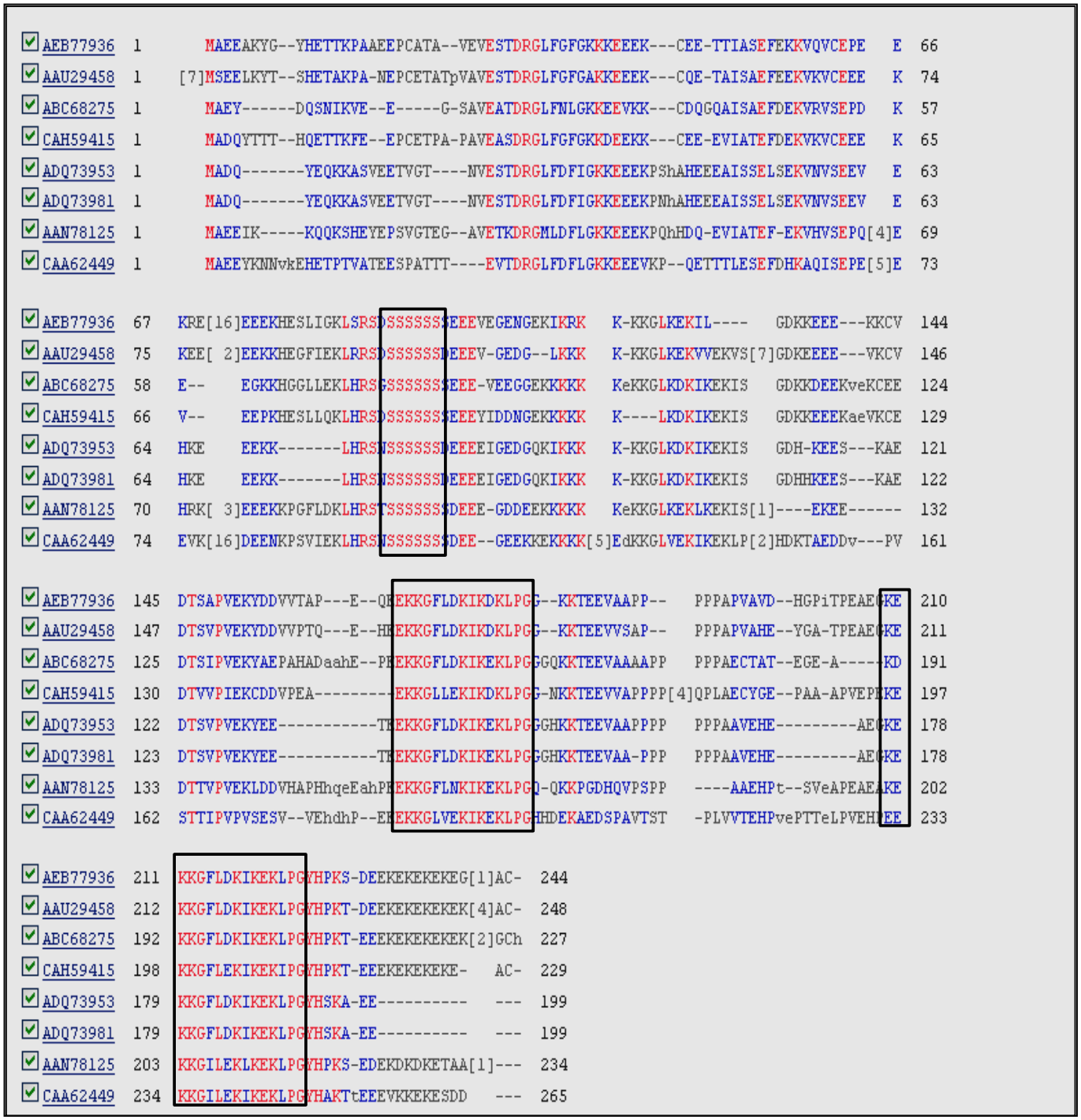


Figure 22: Multi- alignment of sage dehydrin 's deduced protein with other reported dehydrin plant proteins in NCBI database (AEB77936.1: dehydrin [*Salvia officinalis*], AAU29458.1 dehydrin [*Salvia miltiorrhiza*], ABC68275.1: dehydrin [*Coffea canephora]*, CAH59415.1: dehydrin 1 [*Plantago major*], ADQ73953.1: dehydrin [*Solanum chilense*], ADQ73981.1: dehydrin [*Solanum peruvianum*], AAN78125.1: dehydrin [*Citrus x paradisi*], CAA62449.1 dehydrin [*Arabidopsis thaliana*]. SoDNH shares the typical conserved sequence motifs K- and S-segment (bold boxed).

4.1.3.4. The theoretical molecular mass of SoDHN

The calculated molecular mass of SoDHN is 26.96211 kDa using Protein Information Resource website which also confirmed by Compute pI/Mw website (see III.1.9). The isoelectric point was estimated to be 5.27 suggesting that this protein belongs to the class of acidic dehydrins (Figure 23). The amino acid sequence analysis also revealed that glutamic acid represents the amino acid with the highest frequency about 48 glutamic acids are distributed throughout the total amino acid sequence of 244 amino acids corresponding to a relative frequency of 19.7 %. Followed in frequency by lysine, proline, glycin, alanin, valine (Table 2).

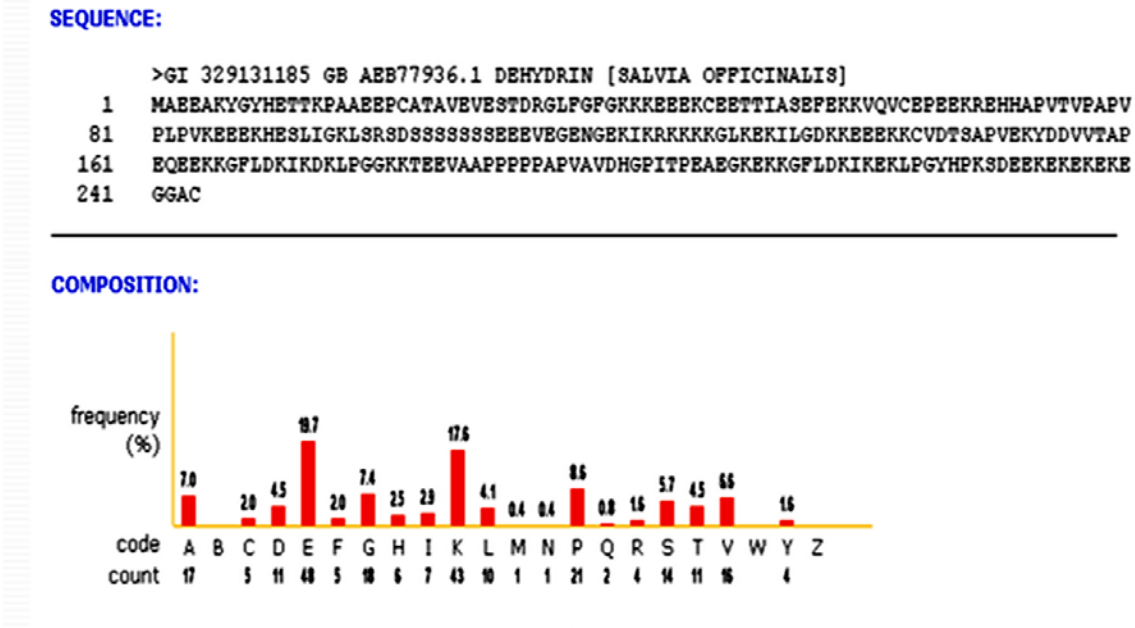


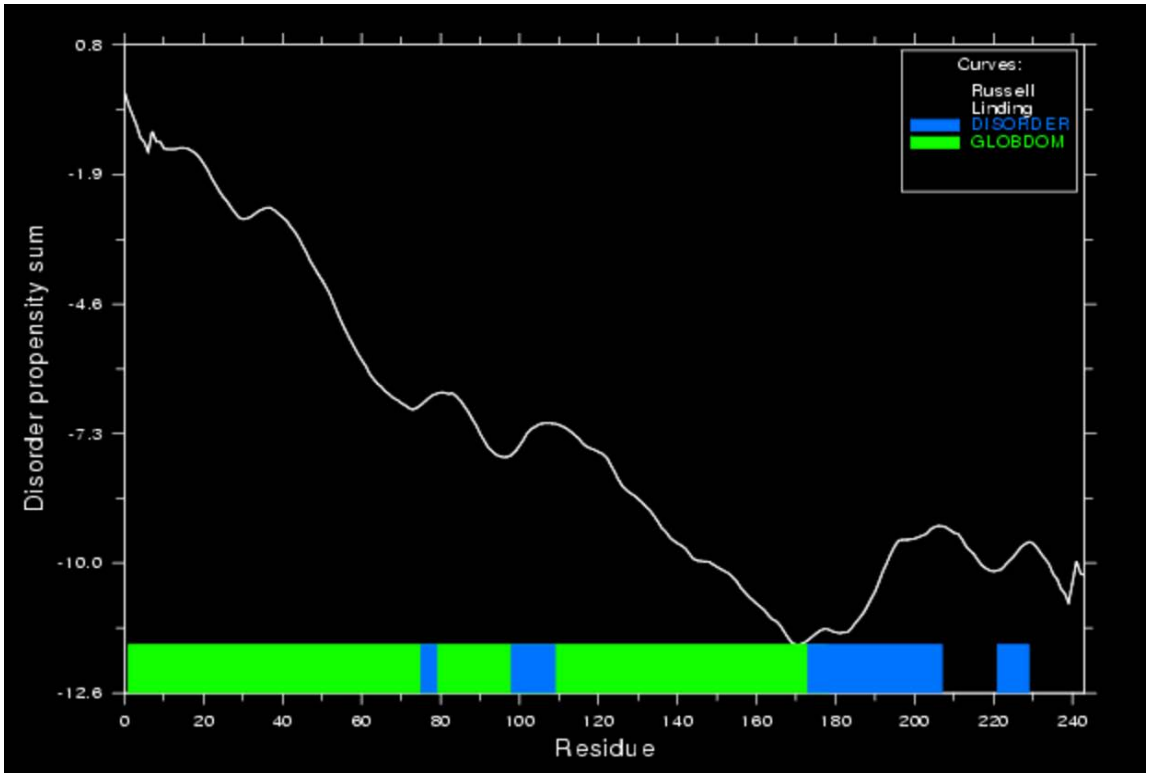
Figure 23: The calculated molecular masses of SoDHN and amino acid sequence analysis

Amino acid	Properties	number	frequency
E (Glu): glutamic acid	Negative charged amino acid	48	19.7 %
K (Lys): Lysine	Positive charged amino acid	43	17.6 %
P (pro): proline	Hydrophobic amino acid	21	8.6 %
G (Gly): Glycin	Hydrophobic amino acid	18	7.4 %
A(Alanin)	Hydrophobic amino acid	17	7.0
V(Val) Valine	Hydrophobic amino acid	16	6.6 %

Table 2: SoDHN amino acids composition (the first six amino acids with the highest frequency).

4.1.3.5. Intrinsically unstructured (disordered structure) of SoDHN

Predication using the computer program Disordered (see III.1.9; Linding *et al*., 2003) revealed that SoDHN is partially unstructured. The disordered amino acid sequences reach from the positions 75 to79, 98 to 109, 173 to 207 and 221 to 229 (Figure 24). Remarkably, these amino acids are rich in glutamic acid, lysine, serine, proline, alanine and arginine. These results are in close agreement with those obtained by Dunker *et al*. (2002)**,** who demonstrated that intrinsically unstructured proteins reveal a distinct amino acid composition, which is characterized by disorder-promoting amino acids (e.g. alanine, arginine, glycine, glutmine, serine, proline,glutamic acid and lysine) and by a low level in order-promoting amino acids (e.g. trptophan, cysteine, phenylalanine, isoleucine, tyrosine, valine, leucine and asparagine).



MAEEAKYGYHETTKPAAEEPCATAVEVESTDRGLFGFGKKKEEEKCEETTIASEFEKKVQVCEP
EEKREHHAPVTVPAPVPLPVKEEEKHESLIGKLSRSDSSSSSSSEEEVEGENGEKIKRKKKKGL
KEKILGDKKEEEKKCVDTSAPVEKYDDVVTAPEQEEKKGFLDKIKDKLPGGKKTEEVAAPPPPP
APVAVDHGPITPEAEGKEKKGFLDKIKEKLPGYHPKSDEEKEKEKEKEGGA C

Figure 24: Disordered structure of SoDHN. Predication using computer program (Disordered by Russell/Linding definition). The disordered amino acid sequences reach from the residues 75 to79, 98 to 109, 173 to 207 and 221 to 229 (blue highlighted).

In conclusion, all the features of SoDHN dehydrin are consistent with the general characteristics of dehydrins as described by Close *et al*. (1996, 1997). Yet, the question arose what are the differences between this dehydrin and dehydrin-like proteins.

4.1.3.6. Alignment between dehydrin-like protein and SoDH

The alignment between an approximately 400 bp dehydrin-like protein (ORF plus 3´-untranslated region) and the 912 bp SoDNH dehydrin (ORF plus 3´-untranslated region) revealed that – apart from the fact that the nucleotides of the SoDHN from 59 to 587 are missing in the dehydrin-like protein - both proteins are almost identical. Only at position 58, thymine (T) in the dehydrin-like protein is exchanged by cytosine (C) in the SoDNH, and the nucleotide sequence (GTACG) before the poly A sequence is missing in SoDHN (Figure 25).

```
DH-like 1   ATGGCCGAAGAAGCGAAATACGGTTACCACGAGACCACCAAGCCCGCCGCCGAGGAGT      58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SoDHN    1  ATGGCCGAAGAAGCGAAATACGGTTACCACGAGACCACCAAGCCCGCCGCCGAGGAGC      58

SoDHN   59  ..........................................................    587

DH-like  59 CGTCGATCACGGCCCCATCACTCCGGAGGCGGAGGGAAAGGAAAAGAAGGGTTTCTTGGA  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SoDHN   588 CGTCGATCACGGCCCCATCACTCCGGAGGCGGAGGGAAAGGAAAAGAAGGGTTTCTTGGA  647

DH-like 119 TAAGATCAAGGAGAAACTGCCTGGCTACCACCCCAAGAGTGATGAGGAGAAGGAAAAGGA  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SoDHN   648 TAAGATCAAGGAGAAACTGCCTGGCTACCACCCCAAGAGTGATGAGGAGAAGGAAAAGGA  707

DH-like 179 GAAGGAAAGGGAGGGTGGTGCATGCTAAGTTAATAATCAAGTCAAGTGGGGTTTGTTGAT  238
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
SoDHN   708 GAAGGAAAAGGAGGGTGGTGCATGCTAAGTTAATAATCAAGTCAAGTGGGGTTTGTTGAT  767

DH-like 239 TATATTTTGTGATTTTCAAGTGTTATTTGTTGATGCTTTTCCAATGTTTCCTATTTTCCC  298
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
SoDHN   768 TATATTTTGTGATTTTCAAGTGTTATTTATTGATGCTTTTCCAATGTTTCCTATTTTCCC  827

DH-like 299 ACTTTTGCTACTTTGTTAATTAAGTTGTATTTGGGGTTTTCATGAATTACATATGTGTAT  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SoDHN   828 ACTTTTGCTACTTTGTTAATTAAGTTGTATTTGGGGTTTTCATGAATTACATATGTGTAT  887

DH-like 359 GTGCAGTCTTTTGTGTACGaaaaaaaaaaa  388
            |||||||||||||      ||||||||||
SoDHN   888 GTGCAGTCTTTTGT-----AAAAAAAAAAA  912
```

Figure 25: Alignment between 389bp fragment and SoDNH at DNA nucleotide level (the differences are yellow highlighted).

In contrast to the SoDHN, the dehydrin-like protein lacks most of the typical dehydrin features. Similar results had been obtained by Schneider *et al*. (1993)**,** who isolated a dehydrin-like protein from *Craterostigma plantagineum*, which accumulates in the chloroplast in response to ABA. Accordingly, with respect to the scope of this study to adopt the expression of dehydrins as marker for drought stress, all further investigations will be focussed on the expression of SoDHN.

4.1.3.7. Phylogenetic classification

Phylogenetic unrooted tree are based on sequence similarity rather than morphologic characters. The phylogenetic relationship of SoDHN with other closely related dehydrin proteins was obtained from the NCBI database. Sequences were aligned using the MUSCLE software (v3.7), which is configured for highest accuracy (MUSCLE with default settings).The neighbour-joining trees were constructed using usual bootstrapping procedure, which is replaced by a new confidence index that is much faster to compute (Figure 26);(Dereeper *et al*., 2008, 2010; Edgar, 2004)**.**

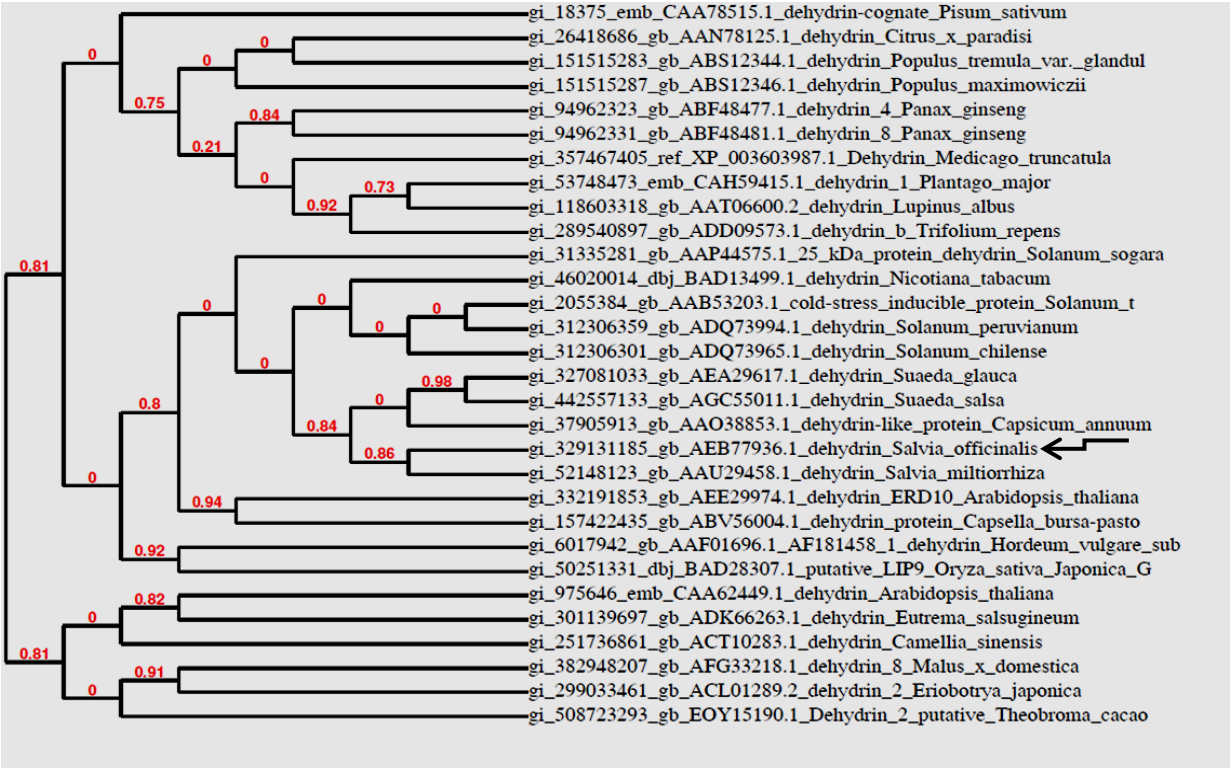


Figure 26: Phylogenetic tree of SoDHN with other closely related dehydrin proteins. The accession numbers of the protein sequences used for building up the phylogenic tree are given alongside. SoDHN is arrowed.

The SoDHN phylogenetic tree proposed that SoDHN is relatively close to plants group belonging to the same family compared to those belonging to quite different families. For instance, plants belonging to the family Lamiaceae (*Salvia miltiorrhiza*) formed in the same clade. However, different families such as Solanaceae *(Capsicum annuum),* Amaranthacea*e* (*Suaeda salsa*), Brassicaceae (*Arabidopsis thaliana*) formed in a separate sub-clade.

Discussion:

Structure and general properties

As mentioned above, dehydrins generally are classified by a highly conserved K-segment which may be present in one or several copies in the sequence. As well, many dehydrins contain a track of serine residues (the S-segment) and/or the Y-segment with the consensus motif (T/VDEYGNP), but they lack in cysteine and tryptophan residues (Allagulova *et al*., 2003; Rorat, 2006). Some dehydrins also reveal a less conservative domain of Gly and polar amino acids named ø-segment (Close *et al*., 1989; Close, 1996).The dehydrin gene from *Salvia officinalis* possesses two K-segments with the consensus EKKGfLDKIKEKLPG motif and an 8-serine residue stretch upstream of the first K-segment.

At least one conserved K-segment is obligated for all dehydrins. This unique motif bears a resemblance to a lipid-binding class A2 amphipathic α-helical segment found in apolipoproteins (one of the plasma lipoprotein components). Its putative function is related to a facilitation of the transport of water-insoluble lipids into the plasma (Close, 1996; Velten and Oliver, 2001).

The SoDHN shares typical characteristics with the members of the dehydrin family, such as the highly conserved lysine-rich domain K-segment and the serine residues S-segment which was described by Close (1996). However, in contrast to dehydrins from many plant species, which contain high percentages of glycine, the deduced amino acid sequence of SoDHN is rich in the charged amino acid glutamate (about 19.7%) , which is more abundant than lysine (17.6%) . Up to now, there are only few dehydrins reported, which are rich in glutamic acid, i.e. those from *Arabidopsis* and wheat (Danyluk *et al*., 1994; Alsheikh et al., 2003). The molecular function of glutamic acid residues in the dehydrin with respect to their significance environmental stress responses are poorly understood. Indeed, glutamic acid as acidic amino acid introduces negative charges. Accordingly, it could be deduced that it may provide higher hydrophilicity to the

dehydrin protein, which, in fact, could enhance its water-binding activity to ensure the maintenance of adequate water in dehydrated cells (Personal communication with Prof. Hara Shizuoka University, Japan).

Furthermore, SoDHN does not share the general tendency of dehydrins to be free of tryptophan and cysteine. Indeed, it lacks tryptophan, but it contains about 2.0% cysteine. SoDHN does not have the conserved Y- motif that is present in several known dehydrins; however, there are some examples of dehydrins lacking this conserved motif. For instance, Rorat *et al*. (2004) reported a KS-type dehydrin in *Solanum* species and Shekhawat *et al*. (2011) isolated a SK_3-type dehydrin from banana.

According to sub-classification of dehydrins into Y_nSK_2, Y_2K_n, K_n, K_nS, and SK_n proposed by Close (1996; 1997), the deduced amino acid sequence of SoDHN reveals that it possesses two K-segments and one S-motif. Thus, the SoDHN belongs to the SK_2 subclass. The subclass of SK_n type is classified as acidic dehydrins (Allagulova *et al*., 2003). The calculated isoelectric point of SoDHN protein is 5.27 signifying that it belongs to the class of acidic dehydrins. These results are in line with Bae *et al*. (2009) who isolated a SK_2-type dehydrin gene from poplar with p*I* 5.14.

It seems that the feature of dehydrins being rich in glutamic acid is correlated with the dehydrin SKn type. Up to now, there are only limited studies reporting dehydrins with high frequency of glutamic acid. However, there is no studies elucidated the function of glutamic acid in the dehydrin protein .

Subcellular localization and phosphorylation

Also with respect to the subcellular localization, the findings for different dehydrins are ambiguous. In general, nuclear localization requires a corresponding signal, usually a short stretch of consecutive basic amino acid residues containing one or more short sequences of positively charged lysine or arginine residues which are exposed on the protein surface (Jans *et al*., 2000; Lange *et al*., 2007; Nair, 2003). Indeed, the SoDHN possesses a serine cluster (S-segment) followed by a lysine motif (KRKKKK) sited at residues 120-126 as a putative consensus site for kinase2 (CK2) phosphorylation, which may function as a nuclear targeting signal sequence (NSL; Figure 21), and thus pointing to a nucleolar localization. The localization

was also supported by prediction of subcellular localization using the PSORT website (prediction of protein sorting signals and localization sites in amino acid sequences).

These results are in accordance with those of Vilardell *et al*. (1990) who showed that maize dehydrin RAB-17 has a potential phosphorylation site and a cluster of serine residues (S-segment) followed by a putative casein-type kinase 2-type substrate consensus sequence. Also Jensen *et al*. (1998) identified the stretch of amino acid residues from 66 to 96, which contains the S-segment and which is adjacent to the protein kinase CK2 recognition site and a sequence of basic amino acids residues (RRKK) sharing similarity to a nuclear localization signal (NLS) of a simian virus 40 (SV40) signal peptide.

However, the subcellular localization of SoDHN needs to be confirmed by additional experiments, applying alternative approaches. The most reliable methods are based on immunological investigations. Yet, such proceeding requires significant amounts of the pure SoDHN protein to raise antibodies. Accordingly, SoDHN was heterologous expressed in E.*coli* (see chapter IV4.3)

Intrinsical unstructuration of dehydrin proteins

Dehydrins are categorized also as a class of Intrinsically Unstructured /Disordered Proteins (IUPs/ IUDPs; Mouillon *et al*., 2006; Eriksson *et al*., 2011; Hanin *et al*., 2011). These proteins have no fixed tertiary structure under physiological conditions. Nevertheless, they play a central role in biology in different cellular processes. i.e., gene expression, cellular signal transduction, and as protein chaperones (Tompa, 2005). Protein disorder can be monitored experimentally by a variety of methods, such as X-ray crystallography, NMR spectroscopy, CD-spectroscopy, and hydrodynamic measurements. Interestingly, there are some computing tools to predict protein structure and function, including identification of those proteins that are partially or completely unstructured (Linding *et al*., 2003).

As mentioned above, SoDHN also shares many of these properties. This is in accordance with the results of Mouillon *et al.* (2008)**,** who examined three dehydrins from *Arabidopsis thaliana* for their intrinsically unstructured feature. The authors used a simplistic model for mimicking cellular dehydration i.e. polyethylene glycol, glycerol, and sugars which plants naturally employ as compatible solutes (i.e. sucrose and glucose). Macromolecular crowding was induced by the large polysaccharides ficoll and dextran. The results showed that dehydrins are extremely stable in their disordered state and that they are only modestly affected by solvent alterations. The same

research group found out that role of the conserved segments is not only to promote the formation of the tertiary structure, but also to apply their biological function more locally upon interaction with specific biological targets. Taken together, these unique features suggest that the dehydrins are highly specialized proteins that have the capability to maintain their disordered character under dehydration conditions.

Conclusion:

To sum up, SoDHN seems to be a typical SK_2 dehydrin which represents a good candidate as appropriate stress marker. Accordingly, based on Real Time PCR its expression was analyzed in response to drought stress.

4.2. SoDHN expression under drought stress

To elaborate the optimal conditions for the estimation of SoDHN gene expression under drought stress, preliminary experiments using detached leaves had been conducted. In a second row of experiments, mimicking the conditions of the *Salvia officinalis* natural environment, the stress related changes in gene expression had been analyzed.

4.2.1. SoDHN gene expression in detached leaves

4.2.1.1. Induction of drought stress by detaching leaves

As described before in the Material and Method section (III. 2.1.2.1), drought stress could be induced by interrupting the water supply of the leaves by detaching them. The study was carried out by analyzing middle aged leaves, 2h, 4h, 6h, 12h and 24h hours after detaching them. The expression pattern was compared with that of undetached leaves (control); after detaching, the leaves were maintained under the same conditions (light and temperature). Water loss and thus the extent of drought stress were estimated by weighing the leaves directly after detaching them and before analysis. As expected, the relative water content declined drastically, after 24h hours about 35% of the initial water content was present (Figure 27).

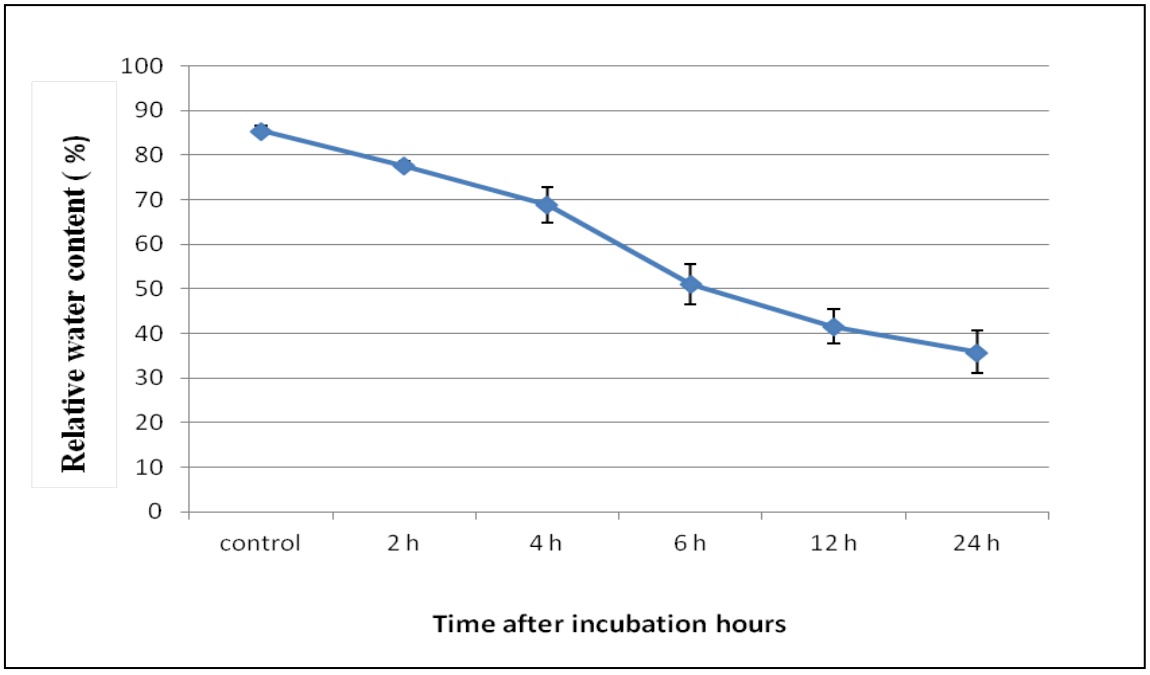


Figure 27: Relative amount of water in detached middle leaves after incubation. Time incubation after hours. Values represent the mean of 2 technical replicates. Error bars represent ± standard deviation.

These data clearly show that detached leaves could be used as an appropriate system to investigate physiological and biochemical changes due to decreasing water potential within a very short time frame.

4.2.1.2. Choosing the appropriate housekeeping genes

Quantitative real time PCR (qPCR) is the most sensitive method for analyzing changes in gene expression. It a reliable and represents the most applied technique in current biological research for quantifying differences in gene expression levels between differentially treated samples. For relative quantification of the expression of a target gene, a comparison to a stable expressed reference genes (internal reference, denoted as housekeeping genes) is required. The major challenge for reliable quantification of gene expression using Real Time PCR is the selection of appropriate housekeeping genes. In this experimental approach six housekeeping genes were tested for a reliable normalization: actin, eF1α[1], α-tublulin, ubiquitin , 18S, and G3PDH[2]). The analyses revealed that 18-S and actin exhibit the most stable expression pattern in the course of stress responses in detached leaves (Figure 28). However, in some experiments, the expression of 18S seems to be slightly influenced by the treatment applied, whereas actin expression turned out to be more stable. Accordingly, the latter was chosen for the further analysis as reference genes and for gaining more reliable results actin and 18S were used (see appendix).

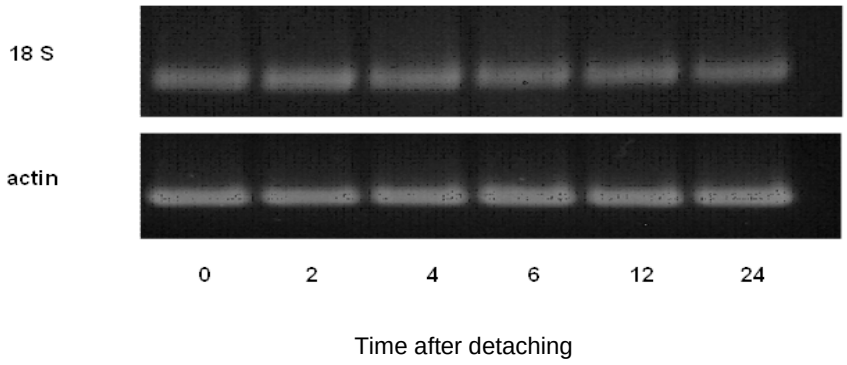


Figure 28: Expression profile of the most stable housekeeping genes (18-S and actin) in detached middle aged leaves.

[1] eF1α corresponds to elongation factor 1α.

[2] G3PDH = Glyceraldehyde 3-phosphate dehydrogenase

4.2.1.3. SoDHN gene expression in detached middle aged leaves

As outlined in the Material and Method section, three primer pairs had been tested to be used for the qPCR, before the system was established. The corresponding analyses revealed that in unstressed leaves, the SoDHN gene was expressed only to a minor extent. The interruption of water supply and thus the presence of drought stress drastically enhanced dehydrin expression (up to 6.5-fold). Astonishingly, the maximum level was reached already after 4 hours after detaching, corresponding to a relative amount of water of about 66 % (Figure 29).

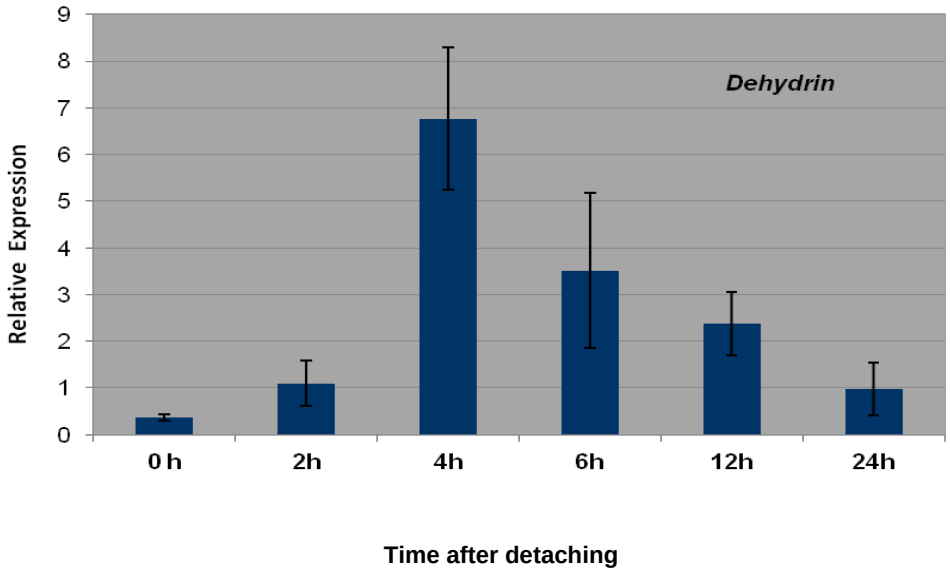


Figure 29: Expression profile of SoDHN gene, in detached middle leaves.Time incubation after hours. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments

Although the water loss in detached leaves is really fast, the very rapid increase in dehydrin expression was unexpected: already after two hours, a significant enhancement was detected and maximal expression occurred already after 4 hours. Obviously, appropriate declines in water content instantaneously triggered the dehydrin expression. Even if the threshold level responsible for the induction of dehydrin expression would be undershot within the first hour, the velocity of the response in gene expression is really exceptional. However, in this context we have to consider that in other experiments analyzing the impact of drought stress on gene expression, water shortage was induced by omitting watering or by applying polyethylene glycol. Accordingly, the time until the related changes in water content of the leaves occurred is much longer. Thus, the observed rapid responses in gene expression in detached leaves are not

necessarily due to a more rapid signaling or signal transduction but obviously are due to the much faster decline in water potential. This finding is confirmed by Yang *et al*. (2012), who analyzed four DHN genes in detached leaves of two grapevine species (*Vitis vinifera* and *Vitis yeshanensis*) in response to drought-rehydration. Maximum level of expression was detected already approximately 2 hours after rehydration of the detached drought stressed leaves.

In addition to the high velocity of changes in gene expression, also the putative down regulation of the dehydrin gene seems to be very unusual. Indeed, if we assume that the increase of dehydrin expression is due to the decline of the water content below a certain threshold, dehydrin expression should remain at the high level. However, we have to consider that the water content drastically decreased further and that after 6 hours already one third of the original water content of the leaves had been gone. It could be assumed that the entire metabolism of the leaves was shut down due to the severe water shortage. Similar observations had been made for the dehydrin expression in coffee seeds by Kramer *et al.* (2010), who demonstrated that the expression of dehydrin gene was triggered by lowering of the water content.

Conclusion: The drought stress induced expression of SoDHN applying RealTime PCR using the elaborated primers and the expression of the both housekeeping genes 18-S and actin as reference genes, represents an appropriate system to monitor drought stress related metabolic changes in sage. Accordingly, this system could be applied to investigate the drought stress responses in sage plants under more natural conditions.

4.2.1.4. SoDHN expression profile in intact plants

Drought stress had been induced by steadily reducing the soil water content. For this, the amount of water daily added was less than that lost by evapotranspiration. A reliable indication that the plants indeed suffer from drought stress, is a partial or complete closure of stomata, visible by a significant decrease in evapotranspiration. Accordingly, the rate of evapotranspiration was monitored throughout the entire experiment.

4.2.1.5. Induction of drought stress and evapotranspiration rate

Drought stress was adjusted to a moderate level, corresponding to 70 to 80% evapotranspiration rate of the well watered control, which is far above the wilting point (Harb *et al*., 2010). As soon as this value was reached, every day the same amount of water that evaporated was added, resulting in a constant drought stress situation all over the entire experiment. It took about four to five days, until the evapotranspiration rate decreased due to the undersupply of water (Figure 30). Apart from the control (taken at the beginning), the first sampling was carried out after 3 days, when the evapotranspiration in the drought stress trial started to decrease. The second samples were taken after 10 days, when the desired rate of drought stress was accomplished (evapotranspiration rate reached 70%). Third sampling was carried out at day 15, when the drought stress persisted already for nearly one week.

The progression of the evapotranspiration rates clearly confirms the postulated course, i.e. after several days of unchanged stomata opening, increasing water shortage induces partial stomata closure. This status could be maintained by the targeted watering regime that exactly compensates the amount of water that was lost by evapotranspiration.

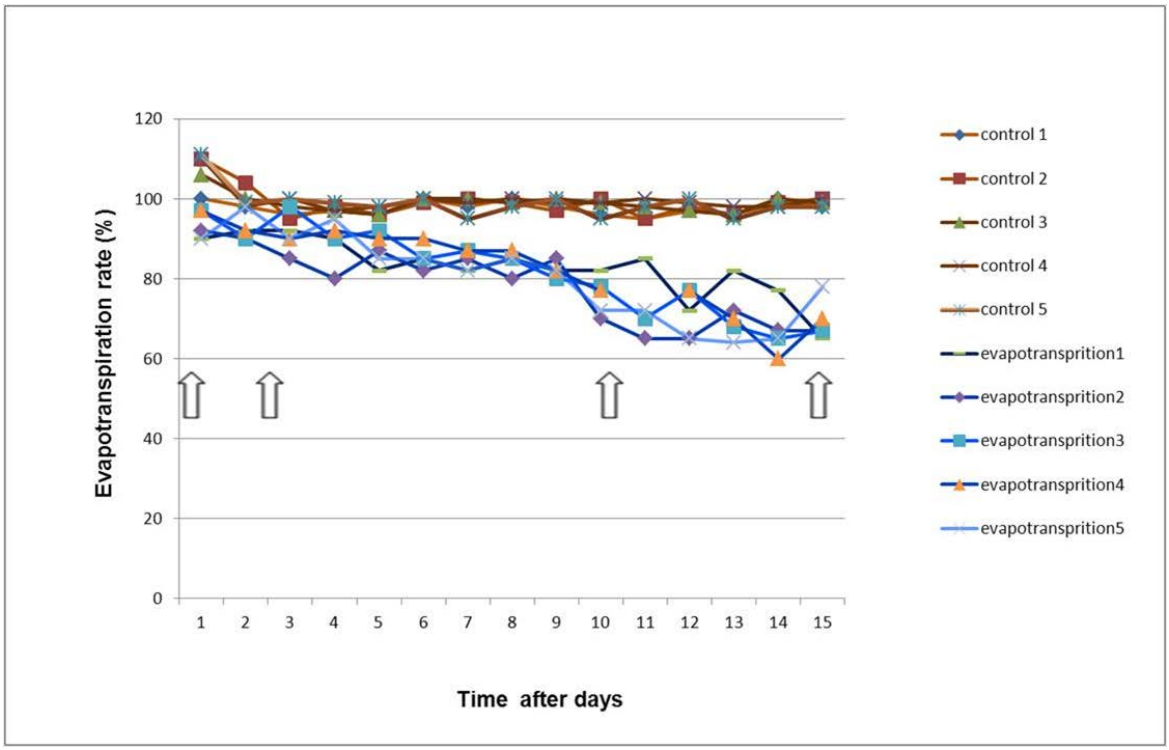


Figure 30: Evapotranspiration rates. Arrows show the sampling points. About 100 sage (*Salvia officinalis*) plants were cultivated under well-water or moderate drought condition. Plants were divided after an acclimatization phase for one week. Drought stress was applied moderate to a half of the plants, while the other half of the plants was cultivated under well-water conditions. For each treatment, samples from three independent plants were pooled to ensure reproducibility of results. Leaves were divided in young and middle aged leaves.

4.2.1.6. Dehydrin expression pattern in leaves of drought stressed sage plants

To estimate the actual stress situation in the drought stressed sage plants, SoDHN expression was determined in young and in middle aged leaves. In both cases, the dehydrin expression followed the same course: in the control plants (before inducing drought stress) SoDHN is constitutively expressed. Three days after the reduction of water supply, when the evapotranspiration rate starts to decrease, no drastic change in dehydrin expression could be detected (Figure 31A&B). In contrast, after 10 days, when the stomata had been closed, dehydrin expression massively increased in young as well in middle aged leaves to up to three fold. Subsequently, when evapotranspiration remained at the low level, and thus stomata are kept closed and drought stress was maintained, dehydrin expression massively declined.

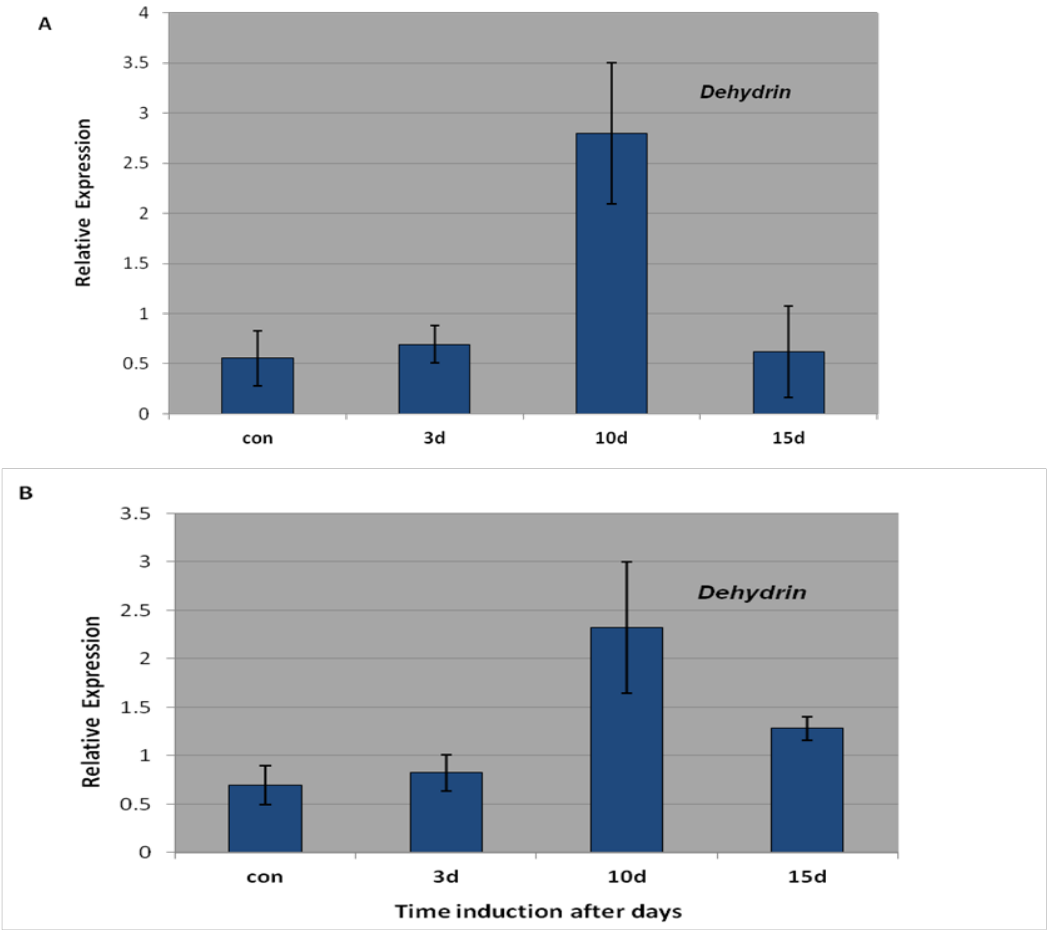


Figure 31: Dehydrin expression in leaves of stressed, intact sage plants. A: young leaves. B: middle aged leaves. Time in days. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments

Discussion:
Drought stress and SoDHN response

Due to the drought related dehydration, the water content in plant cells decreases. As consequence of this water loss which primarily occurs in the vacuole, the cells shrink (Beck *et al*., 2007), membranes are damaged and cellular compartmentalization may be disturbed. In addition to the membrane disintegrety, the increasing of cytosolic viscosity may affect proteins and organelles and reduce their functions (Mahajan and Tuteja, 2005). In consequence, these effects of water deficit strongly reduce the vitality of the cells (Bechtold *et al*., 2013). Apart from these numerous direct impacts of drought related water loss, plants suffer also from indirect effects of water shortage, e.g. due to the drought stress induced stomata closure the supply with CO_2 strongly is reduced. In consequence far less reduction equivalents are consumed and due to the arising over-reduced status toxic oxygen species are produced (for reviews see Wilhelm & Selmar, 2011).

Plants respond to drought stress via several physiological strategies. i.e., at cellular, tissue and whole plant levels enzyme activities are changed, either by activation processes or by the means of changing gene expression. In consequence, plants frequently are able to deal with the stress related harms and are protected against the drought stress (Suprunova *et al*., 2004; Beck *et al*., 2007). In general, stress related changes in gene expression can be divided into early response (within minutes) and late response (within hours). Accordingly, their expression in response to the stress seems to be involved into different stress responses, for example they could be part of certain pathways in responses to the different stressors (Mahajan and Tuteja, 2005; Guo *et al.*, 2009).

One of the major strategies of plants in response to drought stress is the synthesis of hydrophilic proteins (Ingram and Bartels, 1996; Rorat, 2006). These were first observed in cotton during the late stages of embryogenesis; hence they were called LEA proteins (Late Embryogenesis Abundant). They are expressed during the maturation and desiccation phases at the end of seed development (Barker *et al*., 1988). Later on, similar LEA proteins were recognized in the seeds of many higher plants (Ingram and Bartels, 1996; Rorat, 2006). Further analyses revealed that such protein also occur in vegetative tissues, where they in general are up regulated in response to drought and cold stress (Bae *et al.*, 2009). As a kind of preferential hydration mechanism under moderate stress, it is assumed that LEAs wrap interacellular

macromolecules with cohesive water (Hoekstra *et al*., 2001). Under severe dehydration, LEA proteins are thought to act as water replacement agents by exposing their own hydroxylated residues to interact with the surface groups of other proteins (Barker *et al*., 1988). The most prominent LEA proteins are the dehydrins. These small proteins are classified as group II LEA proteins. Typically, they are found in seeds during maturation drying and they are accumulated in dehydrating vegetative tissues, e.g. leaves subjected to environmental stress such as drought, low temperature and salinity (Allagulova *et al*., 2003; Hundertmark and Hincha, 2008).

Although these coherences had been known for a long time, up to recently, there was no approach to outline a linkage between these both areas. A solid alignment of the chain of events leads to the concept that dehydrins in maturing seeds and leaves are part of one basic principle: they are expressed in response to drought stress. A thorough investigation on dehydrin expression in orthodox, intermediate and recalcitrant seeds supports this assumption by unveiling that dehydrins expression and accumulation directly could be attributed to the occurrence or absence of a maturation drying (for details see Radwan *et al*. (2014), section II, Figure 3). Nevertheless, the significance of several classes of dehydrins and the differential gene response to drought stress, salinity or cold and the related physiological functions and biochemical roles, respectively, are not yet fully understood (Hara, 2010).

SoDHN is constitutively expressed

The qRT-PCR data analysis revealed that SoDHN showed a constitutive expression, which, however, is enhanced under drought stress, i.e. in detached leaves as well in leaves of intact plants submitted to drought stress. This increase is in accordance with the finding that dehydrin expression (as group II LEA proteins) is increase during maturation drying. Furthermore, it was reported by several group that dehydrins are up regulated under environmental stresses (i.e., water deficit, high and low temperatures, salinity etc. (Ingram and Bartels, 1996; Bray, 2004 and Wahid & Close, 2007). In contrast, Nylander *et al*. (2001) demonstrated that the expression of some dehydrin is constitutively abundant rather than it is changed in response to stress. Additional study with *Arabidopsis thaliana* also demonstrated that the AtHIRD11 dehydrin is constitutively expressed (Hara *et al*., (2011). Remarkably, its distribution and the accumulation level did not alter when a range of stimuli such as cold, ABA, drought, and NaCl were applied to the plants. This constitutive expression led to the suggestion

that AtHIRD11 may play a role as housekeeping gene. However, it play important role in plant protection under stress (Hara *et al*., 2011). The same suggestion was made by (Mueller *et al*., 2003) who reported that the constitutive expression of the chloroplast dehydrin play a role as housekeeping in the chloroplast. Unfortunately, no conclusive statement could be made, since up to now, no unequivocal evidence for a putative function of dehydrin is available. One of the most discussed putative functions of dehydrins is thought to stabilize membranes and cell integrity. If this indeed is correct, dehydrins first should be expressed in the cytosol rather than in the chloroplast, since this organelle is affected later than the cytosol by unfavourable conditions. If however, the significance of dehydrins is related to the drought stress related impact on photosynthesis, the protective function of dehydrins is indeed necessary in the chloroplasts. To unveil these confusing coherences, much more knowledge on the putative functions of the various dehydrins is required.

Taken together, it could be assumed that during evolution, various subclasses of dehydrins had been developed, e.g. to be expressed as housekeeping genes or to protect against drought stress or photo damage by stabilizing membranes and cell integrity. Thus, their expression should differ in response to the various unfavourable conditions.

Dehydrin subclasses

According to Close (1996, 1997), SoDHN is classified as a SK_2-type dehydrin due to the occurrence of one S-segment and two K-segments in the deduced amino acid sequence. Most SK_2-type dehydrins are expressed constitutively but they are up-regulated under low temperature or in response to drought stress and salt (Rorat *et al*., 2006). In this manner, Bae *et al*. (2009) reported that Podhn, a SK_2 type dehydrin from poplar, is up-regulated in response to drought, salt, cold and exogenous abscisic acid (ABA), whereas wounding and jasmonic acid caused its down-regulation. In contrast to these data, the expression of some other SK_n dehydrins is exclusively induced by low temperatures (for review see; Rorat *et al*., 2006).

Many studies showed that SK_n type dehydrins are principally are located in the vascular tissues of transporting organs (root, stems) and the apical part of the shoots and roots tips as well as in the vascular tissues of leaves (Danyluk *et al*., 1998; Houde *et al* 1995; Nylander *et al*., 2001; Momma *et al*., 1997, 2003). The authors explained this spatial distribution by the fact that

several protective mechanisms for the rapidly dividing and growing cells of these tissues are required already in unstressed situations. Yet, upon cold stress, due to an increasing freeze-induced dehydration, water shortage is expected to be more rigorous for the vascular system and the apical meristems. Accordingly, the presence of SK_n dehydrins is thought to be important for avoiding dehydration in the course of low temperatures. Taken together, SK_n type dehydrins seem to be necessary to protect the transport systems in vascular tissues and meristems already in unstressed plants. However, they are strongly up-regulated by low temperatures presumably to avoid damages by dehydration in the course of cold stress (Danyluk *et al*., 1998; Houde *et al* 1995; Nylander *et al*., 2001; Momma *et al*., 1997, 2003).

Based on the deduced amino acid sequence (Figure 21; 23, Table 2), SoDHN belongs to the subtype of SK_2 dehydrins. Yet, due to some specific characters of SoDHN, it could be deduced that it might reveal some certain functions. In contrast to most other dehydrins which share a glycine-rich repeat that of SoDHN is a glutamate-rich one; and in contrast to the general tendency of dehydrins to be free of cysteine, SoDHN contains 5 cysteines (Figure 23). These properties give hint to the possibility that this SK_2 dehydrin may perform another or additional function, i.e. during drought acclimation. Accordingly, the question arises whether or not SoDHN could also play an important role in preventing potential damages caused by cold stress in *Salvia officinalis*, as it was shown for SK_2 type dehydrins of other plants (see above). This might be of special interest for the further understanding of the role of SK_2 type dehydrins. In contrast to the plants, from which the SK_n type dehydrins had been isolated so far (poplar, wheat, potato) and which are originated from regions with cold winters, *Salvia officinalis* represents a Mediterranean species, which generally is not adapted to withstand cold seasons. Accordingly, it could be assumed that in *Salvia officinalis*, the role of the SK_2 type dehydrin proteins might be shifted from the protection against damages caused by low temperatures to those caused by drought

Comparison between SoDHN expression in response to decreasing water contents in detached leaves and in leaves of stressed intact plants

The dehydrin expression in response to water loss in the detached leaves was very fast and apparently induced by the rapid changes in the leaf water content. In contrast, in the intact sage plants submitted to drought stress, no significant changes in dehydrin expression had been observed within the first three days after drought induction. The leaves are turgescent and any

drastic drought stress related water loss had already occurred to this point. However, in later stages of drought stress, dehydrins, i.e. SoDHN are up-regulated significantly. From this, it could be deduced that, due to the persistent water undersupply, the water content in the leaves decreased very slowly. Obviously, it took several days, until the water concentration in the leaves finally felt below a putative threshold and dehydrin expression strongly was enhanced.

The massive dehydration in detached leaves led to relative water contents of about 50% already after 6h and putatively caused a complete metabolic shutdown in the dying cells. Thus, the decline in SoDHN transcripts in detached leaves might not be caused by deliberate changes in its expression but is due to the dieback of the entire metabolism of the dehydrating cells. Alternatively, the decrease in SoDHN transcripts might also be due to a real down-regulation. However, if we assume that the up-regulation of dehydrins is due to a decline of the water content, the question arises why dehydrin expression in the stressed plants is down-regulated again, although drought stress was maintained. The persistence of the stress situation nicely was documented by the ongoing stomata closure, visible by the unchanged rate of evapotranspiration (Figure 30). If indeed the initial dehydrin up-regulation is caused by a decline of the water content below a certain threshold level, up-regulation should be maintained throughout the entire experiment.

One explanation for this unexpected decrease in dehydrin expression could be an acclimation to the initial stress situation, which finally yields in a maintenance or recovery of the original water content during the course of the persisting stress situation. Yet, in this context, we have to differentiate between “water content” and “water potential”. Whereas the water content indeed could be enhanced again, e.g. by the action of newly synthesised compatible solutes (see below), i.e. by elevating the osmotic potential and thereby increasing the influx of water in the cells, the corresponding water potential, i.e. the availability of water, will further be reduced due the synthesis of osmotic active substances - also when more additional enters the cell.

Another possible explanation for this remarkable down-regulation of dehydrins could be due to the nature of the trigger for the up-regulation. If we assume that not the actual values of water potential (Ψ_w) or water content (θ) and the drop under a certain threshold level is triggering the dehydrin expression, but the slope of its changes ($\Delta\Psi_w$/min; $\Delta\theta$/min) the situation would be quite different. When the plants are exposed to moderate drought stress, the cells will be dehydrated. This means that θ and Ψ_w will change significantly, corresponding to a distinct level

of $\Delta\theta$ and $\Delta\Psi_w$, respectively. However, when the stress situation persists and plants have acclimated to this certain situation, e.g. by stomata closure, at least in the case of moderate drought stress, no further dehydration should occur. This experimentally was achieved by watering the plants with exactly that amount of water, which was lost by evapotranspiration (Figure 30). Thus, within the last five days of the experiment, no changes in water potential (Ψ_w) or water content (θ) occurred, corresponding to $\Delta\theta$ and $\Delta\Psi_w$ values of zero. This is in accordance with the lack of up-regulation of the dehydrins in this period.

In future studies – in addition to the actual values of θ and Ψ_w - also the $\Delta\theta$ and $\Delta\Psi_w$ have to be determined, in order to gain more information about the trigger of dehydrin expression. Yet, such attempt requires that the actual values of θ and Ψ_w have to be determined without destroying the leaves, e.g. by the means of a terahertz device (Breitenstein *et al.*, 2012); otherwise too many plants are required. In a corresponding experiment, the final level of evapotranspiration (e.g. 80% or 60% of the control) should be achieved either fast (e.g. within one week), moderately (e.g. two weeks) and very slowly (three to four weeks). From the daily analyzes of the leaf water status, the $\Delta\theta$ and $\Delta\Psi_w$ values and their alignment to dehydrin expression, it should be possible to decide whether dehydrin expression is triggered by a falling below under a certain threshold level of the water content or potential or by the velocity of their changes, i.e. $\Delta\theta$ or $\Delta\Psi_w$.

For the further detailed discussion of the complex regulation of dehydrin expression and to facilitate the understanding of its multilayered regulation, the initial up-regulation and the subsequent down-regulation are discussed in the following chapters in succession.

Initial up-regulation

Previous studies revealed that ABA is involved in the regulation of dehydrin expression. This mainly was deduced from the finding that the expression of several dehydrins is induced by ABA and that up-regulation of dehydrin expression is correlated with the occurrence of abscisic acid (ABA). Moreover, Shinozaki and Yamaguchi-Shinozaki (1997) demonstrated the presence of potential *cis*-acting DNA elements, known as ABA responsive elements (ABRE) within the dehydrin promoter. In addition, studies by Deng *et al*. (2005) revealed that also the dehydrin promoter gene from *Brassica napus* was ABA-dependent and Thomashow (1993) demonstrated

that the expression of *Arabidopsis* genes encoding the dehydrins COR66, COR47, and COR78 is ABA-regulated, too.

ABA is well known as a second messenger that transmits a ' low water' signal throughout the cell, and also systemically into the surrounding tissues, and thereby promoting cellular and whole plant tolerance against water-deficit stress (Bray, 1993, 2002; Ingram and Bartels, 1996). ABA also is involved in the regulation of stomata closure (Sirichandra *et al*., 2009, Parmentier-Line *et al*., 2002; Olave-Concha *et al*., 2004). In this context, it is considered that the maintenance of the low rate of evapotranspiration means that the stomata had been closed all over the last phase of the experiment. Accordingly, also the ABA concentration in the guard cells must have been at the same high level. As the SoDHN expression was strongly diminished in the last phase of the experiment, while the ABA level should have been constantly high, on the first sight one might deduce that ABA could not be the trigger for the increase in the expression of SoDHN. However, in this connection we have to take into account that increased ABA concentrations required for the regulation of stomata closure only will be present in the guard cells and stomata, whereas it should not be enhanced in other cell types or tissues. Thus, the stress induced transient increase of dehydrin expression indeed could be caused by a corresponding transient occurrence of ABA in other compartments, although ABA concentration in the guard cells was maintained high in the guard cells. Accordingly, we have to consider a special distribution in ABA concentration.

These results fully are consistent with the results of Harb *et al*. (2010) who reported that no significant differences of gas-exchange and stomatal conductance occurred at the late stage of moderated drought stress (after 10 days). Moreover, the expression of ABA dependent ABF3 was only high at the beginning of stress and decreased again. The same authors also found that NCED3, an important enzyme in ABA biosynthesis, was highly induced only at the beginning of stress. Its expression was significantly higher after one day of moderated drought stress compared to that one day before and two or three days after the stress induction. In addition, the same research group found that the common stress responsive cis-elements were enhanced in promoters of genes up-regulated after one day of moderated drought stress and down-regulated after 10 days. These differences in the various ABA related pathways could only be explained if we suppose a special differences in ABA concentration, i.e. high ABA-levels in the stomata throughout the entire period of stress and a transient high level in the other tissues as direct,

temporary short-termed response to the onset of the stress situation. Much more information on this differential pattern and spatial distribution of stress responses are required.

Apart from the transient induction of dehydrin expression by temporarily increased ABA-levels, other dehydrins whose expression is not dependent on ABA, might be also transiently up-regulated after induction of drought stress. Xu *et al*. (2008) observed after applying 5 % polyethylene glycol to *Brassica juncea* L. plants to induce drought stress that the transcript level of the BjDHN2 gene increased continuously during the first 36 h and subsequently decreased at 48 h. A similar transient increase was observed for the BjDHN3 gene, however the time frame differed: the transcript level increased up to 12 h and then declined again after 24 h. Similar results have been obtaind by Labhilili *et al*. (1995), who desiccated seedlings of *Triticum durum*. These authors found a dehydration induced dehydrin gene expression in roots as well as in shoots, which increased to the highest level at 30 h and then declined again after 60 h.

In conclusion, both, types of dehydrins of those being ABA sensitive and those being ABA insensitive are known to be expressed transiently. Thus, up to now, it could not be decided whether or not the expression of SoDHN is dependent on changing ABA levels.

Down-regulation

The question arise, why the dehydrin expression is down-regulated although the stress situation is maintained, i.e. why the dehydrin is expressed only transiently at the beginning of the drought stress, although the water content and the stomata closure remain constant. That indeed dehydrins are up-regulated only within a very short phase of the drought stress, was already demonstrated by Labhilili *et al.* (1995) who showed that dehydrin mRNA expression is transient in the drought-sensitive T. *durum cv. Tomclair*. From this study it could be deduced that the transient expression of dehydrins seems to be related to drought-sensitive plant.

In principle, there are at least two possible explanations for the transient expression of dehydrins in the course of drought stress. Either the dehydrins only contribute to the acclimation of changing stress conditions (i.e. the water content or the water potential) rather than in the ability of the plant to manage persistent stress situations, or the dehydrin proteins are persisting over entire stress situation and no further synthesis is required to establish their protective function throughout the entire stress situation. This however would mean that the dehydrin

protein must be detectable all over the time (e.g. via Western blot analysis, see below). Nevertheless, in both cases the question about the nature of the trigger for the down-regulation remains. As the water content did not change (see above) only alterations in the water potential could be responsible for the shutdown of dehydrin expression. Indeed, it is well known that - as response to various stress situations - the water potential is influenced by osmotic adjustments, involving sugars or other so-called compatible solutes. As result, turgescence of the cell is maintained by increasing the osmotic potential and the water holding capacity (Hoekstra *et al.*, 2001). In this matter, e.g. sucrose synthesis and sucrose-phosphate synthase activity is increased in several plants in response to desiccation to maintain water content of the cells (for review see ; Bohnert *et al.*, 1995; Ingram and Bartels, 1996). With respect to a putative interaction of the water potential and the dehydrin expression, only few data are available: Bravo *et al.* (2001) reported that an increase of sucrose and fructans was accompanied by a decrease of dehydrin expression. Yet, in this context, we have to consider that the accumulation of any osmotic active compounds indeed decreases the water potential. Consequently, if the decreasing water potential will impact on dehydrin expression at all, this should be an up-regulation as it occurs as result of the drought stress induced water loss at the initiation of stress. The only possibility how the water potential might affect dehydrin expression is – as outlined above – thought the velocity of its change. Accordingly, the initial up-regulation of dehydrin expression in the first phase of drought stress should be related to the rate of water loss ($\Delta\theta$) and thus to the celerity of the decreasing water potential ($\Delta\Psi_w$). In contrast, when transpiratory water loss and water uptake via the roots are equated (no further change in stress status, see above), no significant change in the water content of the leaves will occur and the formerly induced up-regulation will be withdrawn.

Another possibility for the observed down-regulation could be due to a negative feedback regulation, i.e. the dehydrin proteins synthesized (as consequence of the up-regulation of the dehydrin expression), will inhibit further expression by counteracting with the related transcription factors. Unfortunately, up to now, no information of such mechanisms are known.

Apart from the molecular understanding of the transient expression of dehydrins, this phenomenon also could be a suitable tool for elaborating more insights into the function of dehydrins. As outlined above, the expression of SoDHN gene is correlated to drops in evapotranspiration. If the relevance of this small protective protein is due to its function in this short phase, in which its RNA is expressed, also the dehydrin protein should be detectable in this

phase and it should be degraded when there is no need for SoDHN anymore. Alternatively, if the protein is not degraded, the initial phase of synthesis could be sufficient to ensure that all over the entire stress situation the protective activity of the dehydrin protein is maintained. Thus, in addition to the expression analyses mentioned, also the pattern of the occurrence of the dehydrin protein has to be determined (see chapter 4.4) in order to get more insights into its function. Moreover, for further elucidation of the function of dehydrins, much more information about the properties of these proteins is required. Therefore, the SoDHN recombinant protein was heterologous expressed in E. *coli* (see next chapter).

4.3. Heterologous protein expression of the SoDHN

As outlined above, one goal of this work was the heterologous expression of the SoDHN recombinant protein in E. *coli* in order to gather more information about the functions of dehydrins. When the recombinant protein was characterized, it turned out that the molecular mass of SoDHN was quite different to the predicted one. Due to this unexpected result, a second approach had been applied using another expression system in order to elucidate the contradictory finding. In the following chapters, the results of both approaches are outlined.

4.3.1. Expression of the SoDHN recombinant protein in *E. coli* using Novagen system

SoDHN gene was successfully sub-cloned from cloning vector pJET1.2/blunt Vector to ligation-independent cloning (LIC) protein expression vector. The open reading frame (ORF) of SoDHN was inserted into directional cloning LIC site using primers (III. 1. 7. 9). (further details are mentioned in Material and Methods). The translation starts by the first methionine of SODHN directly after the His-tag, and the translation stoped by using the stop codon of SODHN. To check the success of transformation, PCR product was separated on 1 % agarose gel (Figure 32 A). Three different E.*coli* host cell (NovaBlue GigaSingles™, BL21(DE3), and BL21(DE3 pLysS (Codon Plus) were used for the transformation.

The recombinant plasmid was successfully over-expressed in the three E. *coli* host strains. After optimization of growth and cultivation conditions, total cell protein, soluble cytoplasmic fractions, and insoluble cytoplasmic fractions have been analyzed in order to choose the most suitable clone. As the transformation of the recombinant plasmid in BL21 (DE3) revealed the highest yields, this system was chosen for further analyses.

The apparent molecular mass of the purified E.*coli* expressed SoDNH was about 54 kDa as estimated by 10 % SDS-PAGE (Figure 32 B). This was quite unexpected, since the molecular mass of the fusion peptide (deduced from the SODHN amino acid sequence of 26.96211 kD, plus 5.7 kDa for the His-Tag is calculated to be about 33 kDa. Therefore, further analyses had been required to confirm that the heterologous protein indeed is the SoDHN, i.e. by MALDI-TOF–MS. For such approach, the expressed His-tagged heterologous protein has to be purified.

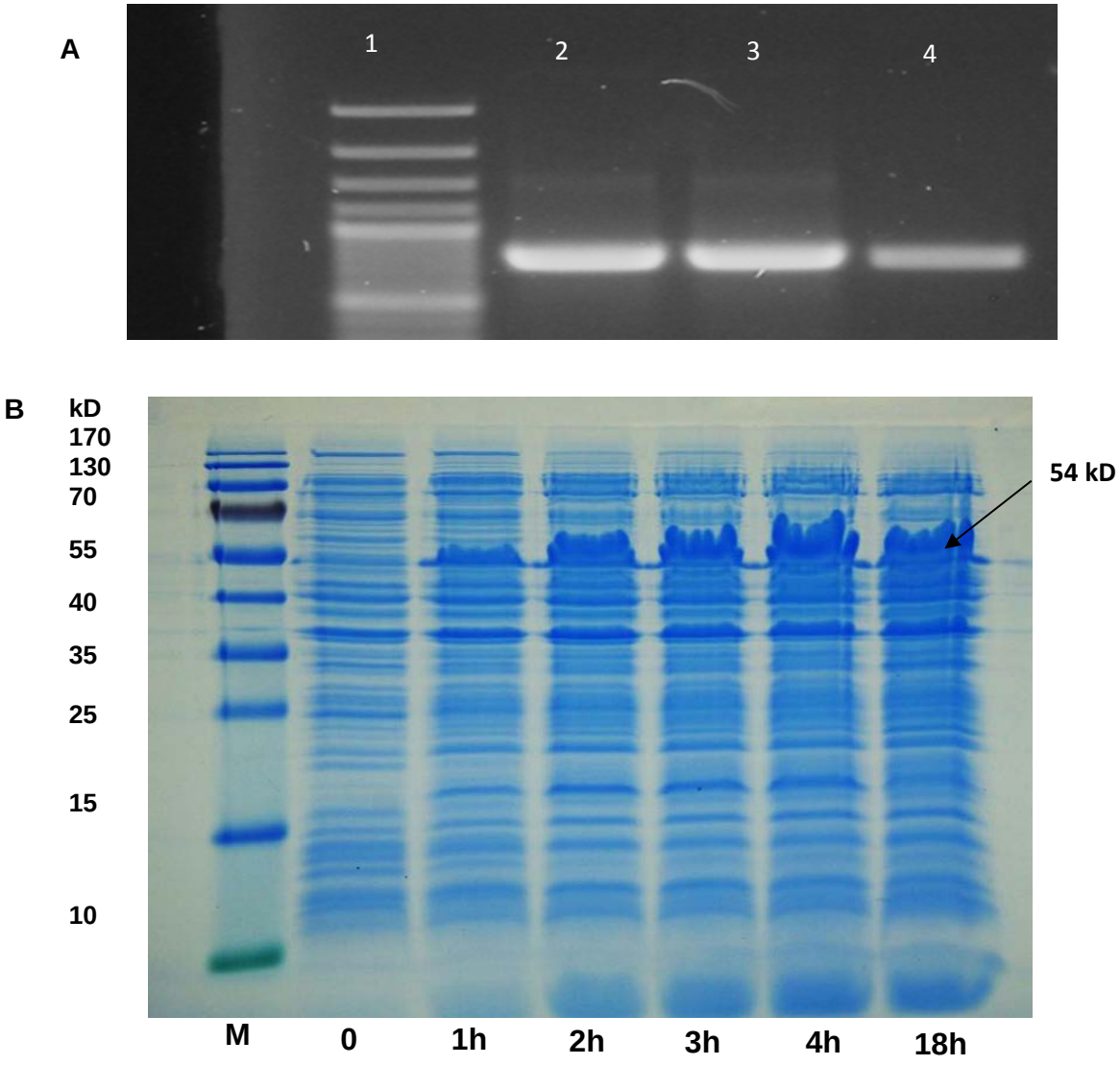


Figure 32: PCR & SDS-PAGE profile. A: PCR for selected clones (lane 1: M, lane 2: recombinant plasmid in NovaBlue GigaSingles competent cells lane 3: recombinant plasmid transformed in BL21(DE3) competent Cells , lane4: recombinant plasmid transformed in (pLysS CodonPlus) competent cells. B: SDS-PAGE of soluble recombinant protein of SoDHN. Lane 1: protein marker, Lane 2: non induced soluble fraction Lane 3:1hour after IPTG induction , Lane 4: 2 hours after IPTG induction, Lane 5: 3 hours after IPTG induction, Lane 6: 4 hours after IPTG induction, Lane 7:18 hours after IPTG induction.

4.3.1.1. Purification of expressed His-tagged heterologous protein

Protein purification of SODHN recombinant protein with His-tag was carried out using a pre-packed HiTrap Chelating column with chelating sepharose high performance (see III. 2.1.4.8). The bound his-tagged protein was eluted by a stepwise gradient of imidazole. The heterologous SoDHN protein was highly eluted in the imidazole fraction 10mM and 20mM (Figure 33) .

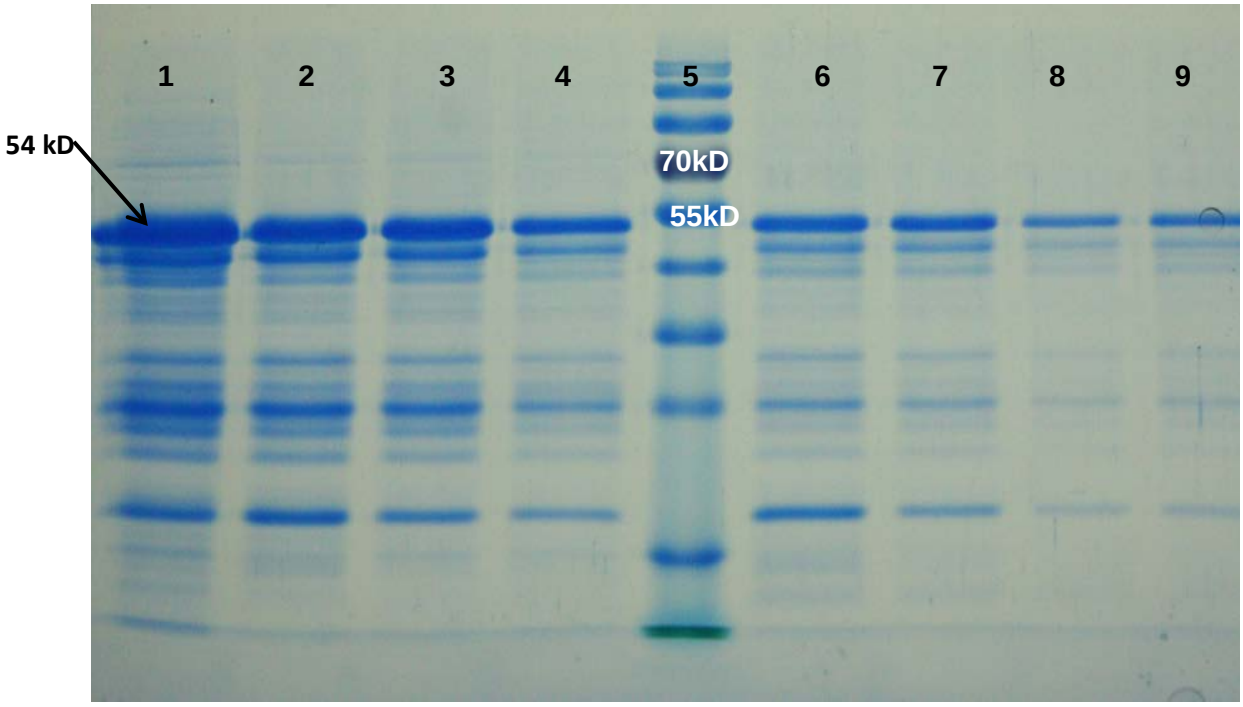


Figure 33: Purification and elution of the SoDHN His-tagged recombinant protein by a stepwise gradient of imidazole (10, 20, 30, 40, 50, 100, 150 mM and 1 M). Lanes from 1 to 4: fractions of 10mM imidazole, Lane 5: protein marker (Fermentas) and lanes from 6 to 9: fractions of 20mM imidazole.

4.3.1.2. MALDI-TOF–MS analysis of the recombinant protein

After electrophoresis, the putative ~ 54 kDa heterologous protein was excised from the gel and dried in a speedVac. The purified heterologous protein was digested with trypsin into short peptides before being immobilized on metallic matrix. MALDI-TOF–MS analysis revealed several peaks related to the dehydrin gene. However, the heterologous protein seems to be conjugated or bound to another unknown protein.

The two longest fragments which seemed to be related to the dehydrin gene were additionally analysed directly by MS/MS. Alignment with the original SoDHN sequence revealed

that the sequences of both fragments (A and B) perfectly match with that of SoDHN. The 26 amino acids of A reveal the same sequence as present in the centre of SoDHN, and that of the 10 amino acids of B is identical to a sequence at the N-terminal of SoDHN protein (Figure 34 A, B).

A

Average Mass = 2912.0980, Monoisotopic Mass = 2910.2977
Residues 1-26
N-Terminus = H, C-Terminus = OH
Modified amino acids: CAM(B) = CAM Cysteine
Fragment ions: Monoisotopic/Average (40000) m/z ratios with 1 positive charge(s).

b	164.071	221.093	384.156	521.215	650.257	751.305	852.353	980.448	1077.500
	1	2	3	4	5	6	7	8	9
	Tyr	**Gly**	**Tyr**	**His**	**Glu**	**Thr**	**Thr**	**Lys**	**Pro**
	26	25	24	23	22	21	20	19	18
y''	-	2748.242	2691.221	2528.157	2391.098	2262.056	2161.008	2059.960	1931.866

b	1148.538	1219.575	1348.617	1477.660	1574.713	1734.743	1805.780	1906.828	1977.865
	10	11	12	13	14	15	16	17	18
	Ala	**Ala**	**Glu**	**Glu**	**Pro**	**CAM**	**Ala**	**Thr**	**Ala**
	17	16	15	14	13	12	11	10	9
y''	1834.813	1763.776	1692.739	1563.696	1434.653	1337.601	1177.570	1106.533	1005.485

b	2076.934	2205.976	2305.045	2434.087	2521.119	2622.167	2737.194	-
	19	20	21	22	23	24	25	26
	Val	**Glu**	**Val**	**Glu**	**Ser**	**Thr**	**Asp**	**Arg**
	8	7	6	5	4	3	2	1
y''	934.448	835.380	706.337	607.269	478.226	391.194	290.146	175.120

The 26 amino acids of A are identical to a sequence at the N-terminal of SoDHN protein:

YGYHETTKPAAEEPCATAVEVESTDR

B

Average Mass = 1273.4332 Monoisotopic Mass = 1272.6132
Residues 1-10
N-Terminus = H, C-Terminus = OH
Modified amino acids: CAM(B) = CAM Cysteine
Fragment ions: Monoisotopic/Average (40000) m/z ratios with 1 positive charge(s).

b	100.076	228.135	327.203	487.234	616.276	713.329	842.372	971.414	1099.509
	1	2	3	4	5	6	7	8	9
	Val	**Gln**	**Val**	**CAM**	**Glu**	**Pro**	**Glu**	**Glu**	**Lys**
	10	9	8	7	6	5	4	3	2
y''	-	1174.553	1046.494	947.426	787.395	658.352	561.300	432.257	303.214

b	-	
	10	
	Arg	
	1	
y''	175.120	

The 10 amino acids of B reveal the same sequence as present in the middle of SoDHN:

VQVCEPEEKR

putatively might reveal only low affinity to bind SDS (personal communication from Prof. Hara, Shizuoka University, Japan). Yet, when less SDS binds to SoDHN, the protein does not have the same strong negative charge as other proteins of the same size. Accordingly, SoDHN does not

migrate that fast in the SDS-PAGE as the other proteins. This assumption first was formulated within an early study by Panyim and Chalkley (1971), who proposed that dehydrins rich in lysine exhibit abnormal motilities in the SDS-PAGE gel. These coherences were confirmed by Hara *et al*. (2013), who also reported that dehydrins show irregular mobility in electrophoresis. However, there have also been contrary finding reported in the literature, in which the calculated molecular masses of the recombinant dehydrin protein indeed are in agreement with apparent molecular mass in SDS-PAGE gels (Hara *et al*., 2011).

As second possibility for the observed differences it could be assumed that the authentic protein indeed reveals 33 kD (as it was calculated from the amino acid sequence deduced from the c-DNA of SoDHN, but that the mass of the recombinant SoDHN protein in fact is higher. This, however leads to the question what is the reason for the enhancement of the molecular mass of the recombinant SoDHN (apart from the 5.6 kD for the His-Tag of fusion peptide).

One possibility could be due to the fact that the recombinant protein strongly binds to a protein from *E. coli*, i.e. SoDHN protein is interacting with an unknown protein**.** Alternatively, we have to assume that the SoDHN is indeed larger that the predicted protein. This could be due to the fact that the stop codon of SoDHN didn't work and accordingly, since there is no additional stop codon (see, III. 2.1.4.1) in the construct LIC expression vector, translation continued. As feasible approach to further elucidate these options, another expression system was applied.

4.3.1.3. Cloning of SoDHN gene using Invitrogen system

The construction of SoDHN/pRSET B plasmid was started by introducing the recognition sequence sites of the restriction enzymes Bam H1 and EcoR1 into dehydrin sequence (see Material and Methods, Table 1.7.10). After successful sub-cloning of SoDHN, the DNA sequence was inserted into the pRSET B plasmid. SoDHN DNA sequence includes an ATG translation initiation codon and a stop codon. The restriction sites were introduced into the sequence to positioned SoDHN DNA sequence downstream and in frame. The translation ended by SoDHN stop codon followed by additional construct stop codon (see III. 2.1.5). This should ensure that translation without fail is terminated after the SoDHN. The plasmid was successfully over-expressed in the *E. coli* strain BL21. The efficiency of over-expression was examined by SDS-PAGE (Figure 35).

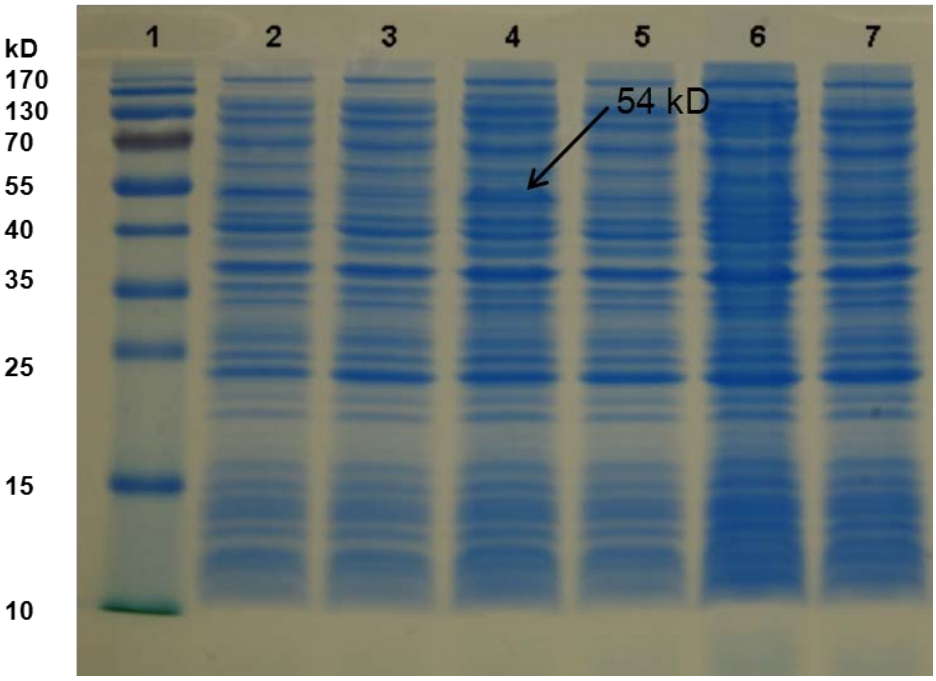


Figure 35: Expression of SoDHN recombinant protein. Lane 1: protein marker (Fermentas), Lane 2: positive control (pRSET,B construct with insert before induction), Lane 3: negative control (pRSET,B construct without insert before induction, Lane 4: Expression construct with insert, 2 hours after induction with IPTG, Lane 5: Expression construct without insert after 2 hours induction with IPTG, Lane 6: Expression construct with insert, 4 hours after induction with IPTG, Lane 7: Expression construct without insert after 4 hours induction with IPTG.

As the SoDHN recombinant protein (expressed as His-fusion protein) reveals a molecular mass of around 54 kD, the results obtained from the Novagen expression system fully had been confirmed. Thus, we can rule out that the high molecular mass of the heterologous expressed SoDHN is due to a malfunction of the stop codon. Accordingly, the only possibility that the observed behaviour in the electrophoresis indeed is due to an enhanced SoDHN protein is due to a putative interaction with an unknown small protein from *E. coli.* Otherwise, it must be due to special, unusual properties of the dehydrin as mentioned above.

4.3.1.4. Heat stability of recombinant SoDHN

In order to get more information on a putative protein-dehydrin-interaction, a special property of dehydrin could be exploited, i.e. the thermal stability of many dehydrin proteins. When these thermo-stable dehydrin proteins are heated in aqueous solution (to 100°C), they

remain stable and soluble in contrast to most other proteins, which will be denatured under these conditions and thereby getting insoluble. The thermo-stability of dehydrins was first reported by by Close *et al*. (1989); and later on confirmed by Close (1997) and Hara *et al*. (2011). Borovskii *et al*. (2002), isolated five putative dehydrins from mitochondria of winter wheat, rye and maize; yet, only two of them were thermo-stable. Due to the high probability that also SoDHN is thermo-stable, a corresponding approach was performed, which was aimed to denature the putative protein interacting with the recombinant SoDHN and thereby preventing further interaction. The soluble protein fraction of the heterologous expression was heated 30 min at 100°C. Denatured protein was separated by centrifugation. SDS was added to the supernatant, before electrophoresis. Astonishingly, the molecular mass of the heterologous dehydrin protein did not change (Figure 36).

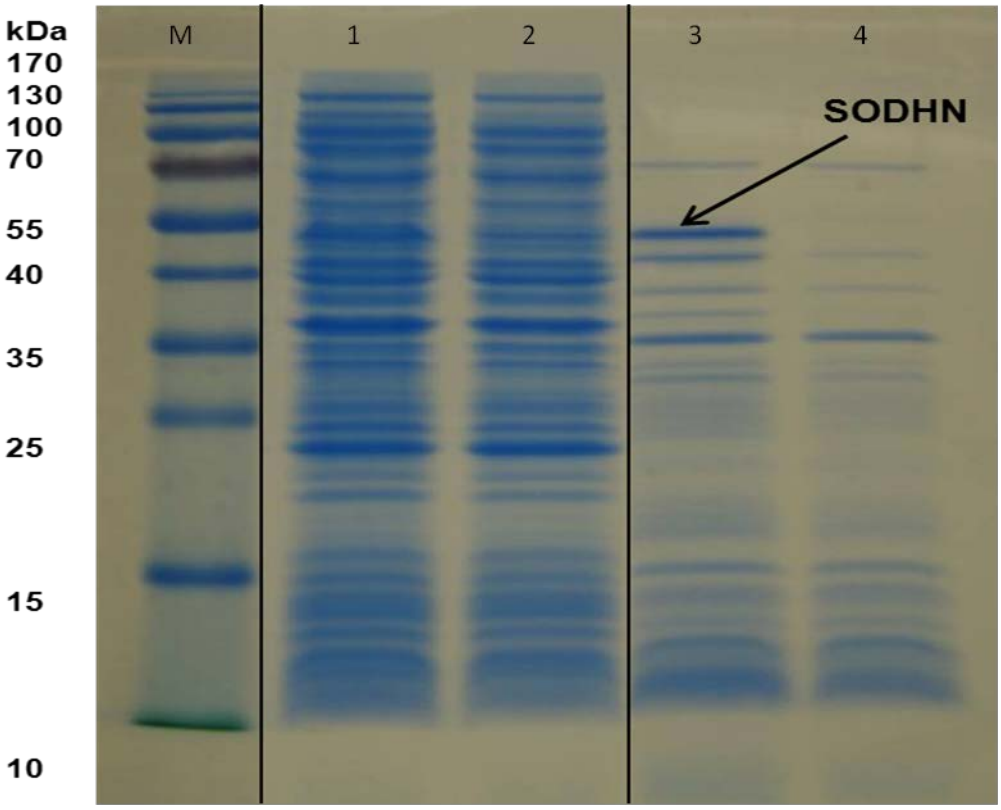


Figure 36**:** Heat stability of recombinant SoDHN. Lane 1: Expression construct with insert, 4 hours after induction with IPTG, Lane 2: Expression construct without insert 4 hours after induction with IPTG, Lane 3: Headed expression construct with insert 4 hours after induction with IPTG, Lane 4: Headed negative control without insert 4 hours after induction with IPTG.

From this result, it has to be deduced that either the protein which is interacting with the dehydrin is thermo-stable, too and thus the interaction with the SoDHN maintained, or that the reason for the slow migration is not due to an enhanced molecular mass, but is due to special, unusual properties of the dehydrin as mentioned above. Yet, it is very unlikely that a certain protein, putatively interacting with the SoDHN, is present in the same large concentration as the strongly expressed dehydrin. Accordingly, apart from the putative protein-dehydrin-complexes, also free dehydrin proteins should be detectable. Indeed, due to their low concentration, by simple protein staining procedure, they might have not been detected so far beside the other proteins present. Thus, Western blot analyses applying dehydrin antibodies[3] have been performed.

4.3.1. 5. Western blot analysis of heterologous SoDHN

For the Westren blot analysis the recombinant SoDHN proteins obtained from both expression systems (vector LIC and vector pRSET B) have been used. The polyclonal antibody, which kindly was provided by Prof. Dr. T. Close (UC Riverside), recognized the K-segment of dehydrins (details are outlined in section III. 2.1.8). It already was successfully used to detect dehydrins in *Arabidopsis thaliana, Pisum sativum, Zea mays, Triticum aestivum, Triticum durum, Hordeum vulgare, Secale cereale* and cyanobacteria (Close and Lammers, 1993; Labhilili *et al*., 1995; Borovskii *et al.*, 2000; Mueller *et al*., 2003; Alsheikh *et al.*, 2005; Kosová *et al*., 2008).

As expected, the antibody recognizes also the recombinant SoDHN (Figure 37 B) and the 54 kD band was confirmed to be the SoDHN. Negative control (without insert – Figure 37, lane 2 and 3) showed no dehydrin signal. Markedly, already in the assay without isopropyl-thiogalactosid (which is required for the induction of the transcription of the vector) SoDHN protein is present. Obviously, there is a basal translation of the introduced vector.

Yet, as most important result we have to state that – apart from the 54 kD signal, no signal for a 33 kD dehydrin is detectable. However, if the 54 kD signal indeed would be due to a complex of an small *E. coli* protein with the 33kD heterologous SoDHN, there should be at least some free dehydrins present. Thus, we can conclude that the apparent molecular mass of the

[3] The dehydrin antibodies used, interact with most dehydrins tested so far.

heterologous SoDHN protein of about 54kD is not due to an interaction with bacterial protein, but must have other causes.

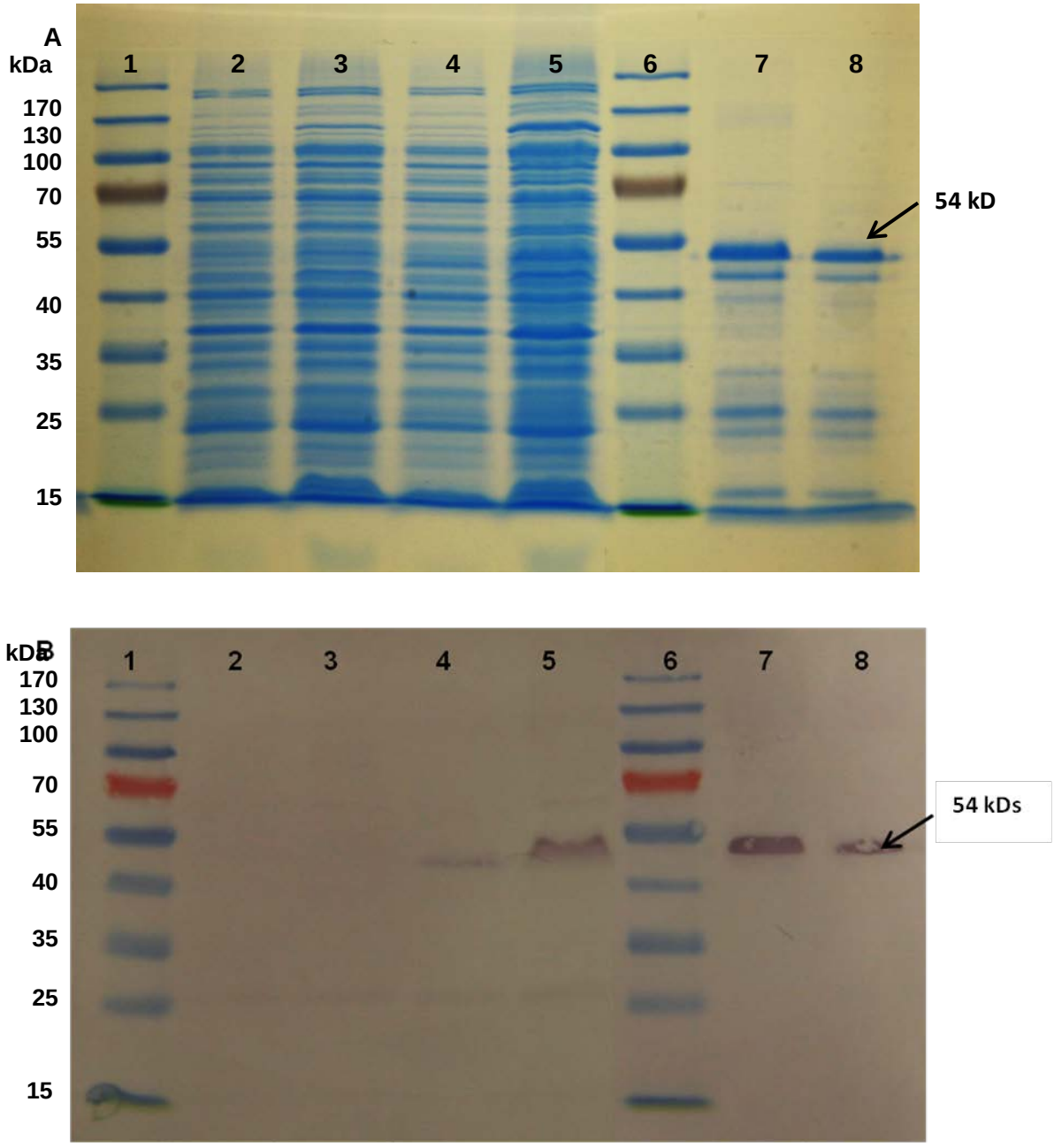


Figure 37: SDS-PAGE & immunoblot analysis of recombinant protein SODHN (~ 54 kDa protein (arrow)). A & B: Lane 1: protein marker (Fermentas), Lane 2: negative control (pRSET,B construct without insert and before induction), Lane 3: negative control (pRSET,B construct without insert 4 hours after induction with IPTG), Lane 4: Expression of pRSET,B construct before induction, Lane 5: Expression pRSET,B construct 4 hours after induction with IPTG, Lane 6: protein marker, Lane 7: imidazole 20 mM fraction (from LIC protein expression vector), Lane 8: imidazole 10 mM fraction(from LIC protein expression vector).

As outlined above, an alternative explanation is based on the assumption that the SoDHN protein reveals special, unusual properties mentioned above. Unfortunately, the elucidation and explanation of this issue becomes much more complicated since the SoDHN protein expressed in the sage plants indeed exhibits a molecular weight of 27 kD (Figure 38). In order to analyse the SoDHN expression in *Salvia officinalis* plants at protein level, SDS electrophoresis of protein extracts from stressed sage leaves have been performed. After Western blot and immunological detection, the SoDHN was visible as dehydrin positive bands at 27 kD. In contrast to the heterologeous SoDHN protein, this molecular mass perfectly fits to the predicted one (calculated on the bases of the SoDHN sequence).

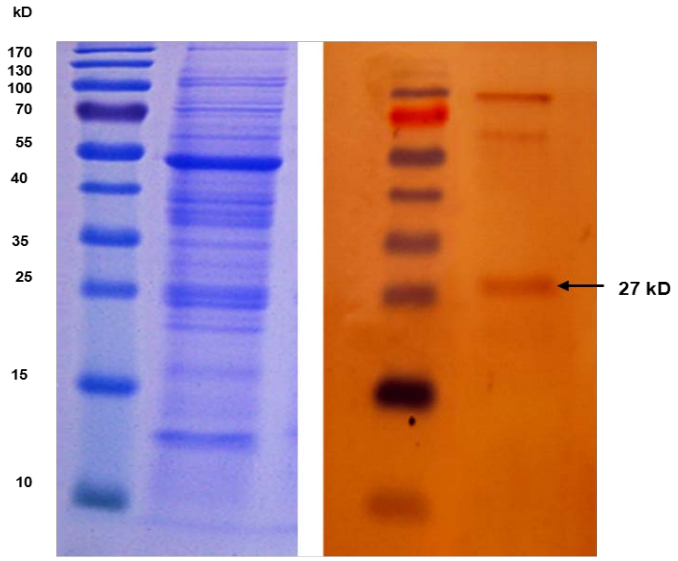


Figure 38: SDS-PAGE & immunoblot analysis of *Salvia officinalis* plant protein. SoDHN is arrowed as visible positive band at 27 kD (arrowed).

The question arose, what are the reasons for the differences in electrophoretic behavior of these both proteins. As already concluded in the last chapter, the interaction with a small protein from *E. coli* could be excluded. However, the interaction of the SoDHN with each other, i.e. to produce dimeric proteins could not be excluded. Up to now, there are only two studies which suggested that dehydrins exist as a dimer (Close, 1996; Lin *et al*., 2012). Therefore, one may speculate that the recombinant SoDHN is a very stable-bridged dimer of two identical dehydrin monomers. To elucidate this discrepancy further experiment should be performed such as gel filtration. However, dehydrin polyclonal antibodies represent a suitable tool for the estimation of dehydrins in sage plant.

4.4. SoDHN expression in *Salvia officinalis* at protein level

As mentioned before, the expression of SoDHN at mRNA level in stressed plants (see 4.2.1.6) declined again, although evapotranspiration was decreased and thus the stress situation was maintained. The question arose, why SoDHN expression is down regulated despite the fact that the plants still suffer moderate drought stress. One obvious reason could be due to the fact that the dehydrin protein arising from the temporal expression of SoDHN at mRNA level will be retained throughout the entire stress situation.

To answer this question, proteins from sage leaves exposed to drought stress were extracted in parallel to RNA isolation. Extracted proteins were analyzed by SDS- polyacrylamide gel electrophoresis (Figure 37; A) followed by immunoblotting using K-segment polyclonal antibodies (Figure 37; B), which already had been successfully used to investigate the differences between the genuine and the heterologous expressed protein (see above).

Western Blot analysis revealed unexpected results. The SoDHN protein (27 kD) is already present in non-stressed leaves. Whereas the basic level is relatively low in elder leaves, it is markedly in young ones. Drought stress significantly enhances the concentration of SoDHN protein in young leaves. This enhancement should be due to the temporal up-regulation of SoDHN on RNA level. When this up-regulation is retracted, no further proteins are produced; however, due to its high stability the enhanced level of SoDHN protein is maintained. Obviously the SoDHN has a long half lifetime of more than several days.

Apart from the SoDHN two other putative dehydrin proteins could be ascertained on the basis of the dehydrin K-segment specific antibody; corresponding signals appeared at 100 and at 65 kD. This is not surprising, since this antibody is known to interact with nearly all dehydrins known so far (Close and Lammers, 1993; Labhilili *et al*., 1995; Borovskii *et al.*, 2000; Mueller *et al*., 2003; Kosová *et al*., 2008) and most plants are described to reveal more than one dehydrin

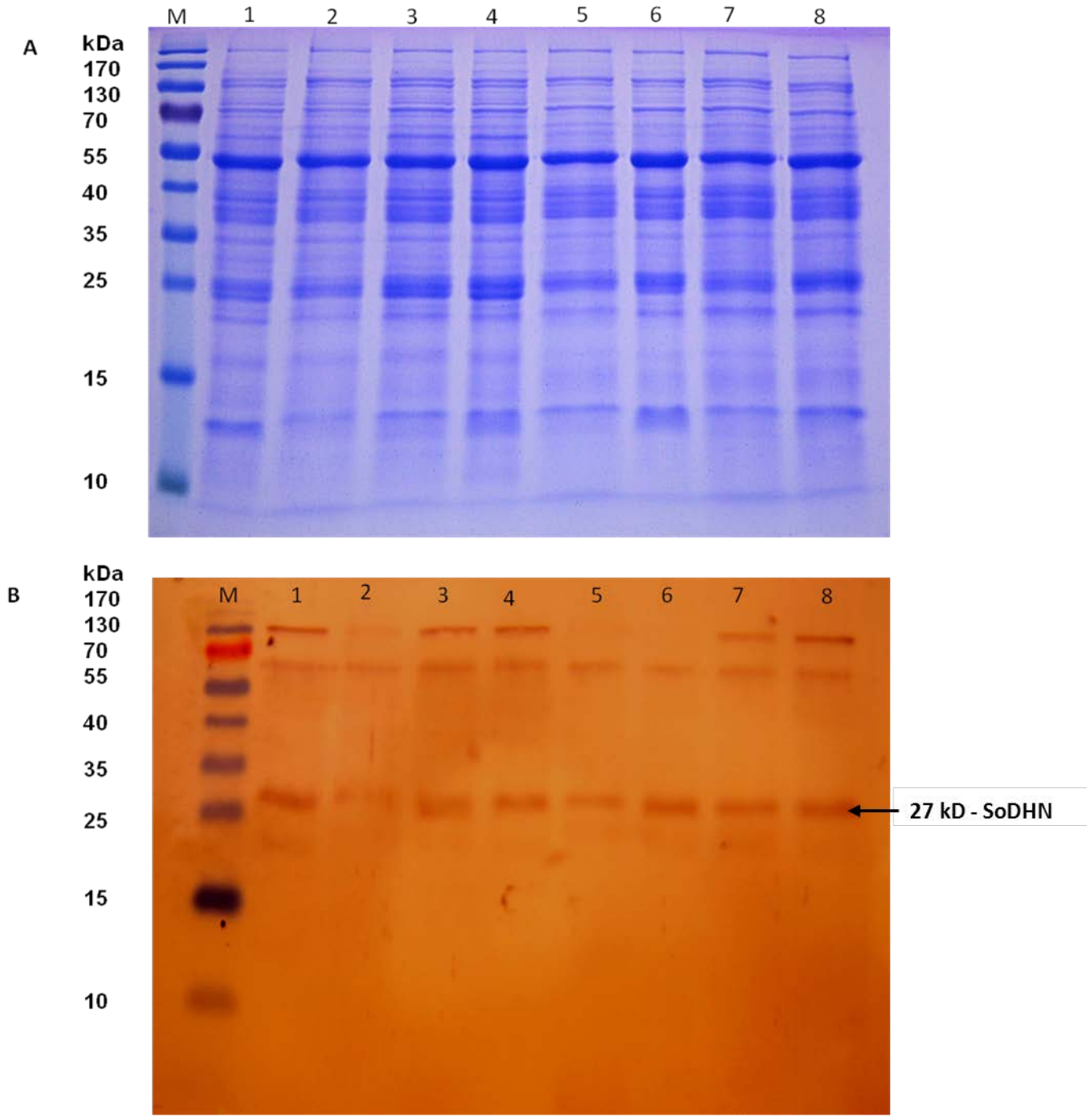


Figure 37: Dehydrin expression in leaves of stressed intact sage plants; A: SDS-PAGE & B: immunoblot analysis. M: protein marker (Fermentas), Lanes from 1 to 4: control, 3 days, 10 days and 15 days respectively, of young leaves after drought stress induction, Lanes from 5 to 8: control, 3 days, 10 days and 15 days respectively, of middle aged leaves after drought stress induction.

(Nylander *et al*., 200; Olave-Concha *et al.*, 2000). Whereas there are no differences in the content of the 65 kD dehydrin, the concentration of the 100 kD dehydrin changes analogously to the SoDHN content.

As shown for the SoDHN, young leaves contain high amounts of the inducible dehydrins (SoDHN and the 100 kD dehydrin), but its concentrations decreased whilst further development (this behaviour was detected repeatedly in all three Western blots performed – data not shown). From this, it could be deduced that this dehydrin is produced in young leaves and during further development, it is degraded. Mature leaves do not reveal this 100 kD dehydrin protein (Figure 37; line 5 and 6). Corresponding developmental related differences in the occurrence of dehydrins are also known for *Trifolium repens* (Vaseva *et al*., 2014) or *Solanum tuberosum* (Rorat, 2004). However, drought stress increases the expression of this dehydrin, too. Obviously, this protein might be important to attenuate the drawbacks induced by the stress situation. In this context, it is interesting to consider that very young leaves express the 100 kD dehydrin obviously is constitutively occurring. Accordingly, we could assume that in young leaves – may be due to the fact that the photosynthetic apparatus is not yet completely balanced, and therefore already under standard conditions stress-like situations might occur (Baker and Leech, 1977) as compared with these, mature leaves behave different. Obviously they are fully adapted and balanced with respect to their photosynthetic capacity, and thus, a putative protective mechanism by the 100 kD dehydrin is not any more required.

In contrast to the both other dehydrins so far mentioned, the 65 kD dehydrin is constitutively present in all samples. Corresponding finding have been reported by Hara (2011) for the *Arabidopsis* dehydrin AtHIRD11 which acts as a housekeeping gene.

Discussion

Stability of the SoDHN protein

The comparison between the western blot and the real time PCR analysis of SoDHN revealed that the level of SoDHN proteins does not correlate with its expression in the course of drought stress (Figure 38). This confirms the hypothesis that – although the stress related SoDHN expression at mRNA level was only transient (Figure 38) - the related dehydrin protein might accomplish its protective function throughout the entire stress situation. Due to its stability the protein remained at an enhanced level for the entire period when evapotranspiration was

maintained at 70 to 80%, i.e. the plants suffer moderate drought stress. Apart from *Salvia officinalis*, also other plants are known, in which the stress induced dehydrin expression on RNA level did not correlate with the protein content. Parmentier-Line *et al*. (2002) showed that dehydrin protein content in stressed blueberry cell cultures did not follow the transcription rate. An opposite effect was observed by Sun *et al.* (2009)**,** who analyzed the impact of salicylic acid and drought stress on dehydrin DHN5 in leaves of *Hordeum vulgare*. The authors reported that high concentrations of salicylic acid caused a decrease of the DHN5 protein, although the high expression level of the corresponding gene was maintained. From these examples it has to be concluded that not in all cases the expression of dehydrin (on RNA level) is a suitable tool for monitoring the actual stress level. In the case of SoDNH, the transient expression allows to indicate the initiation of the stress situation. However, the monitoring of the actual and ongoing stress level requires the estimation and comparison at protein level.

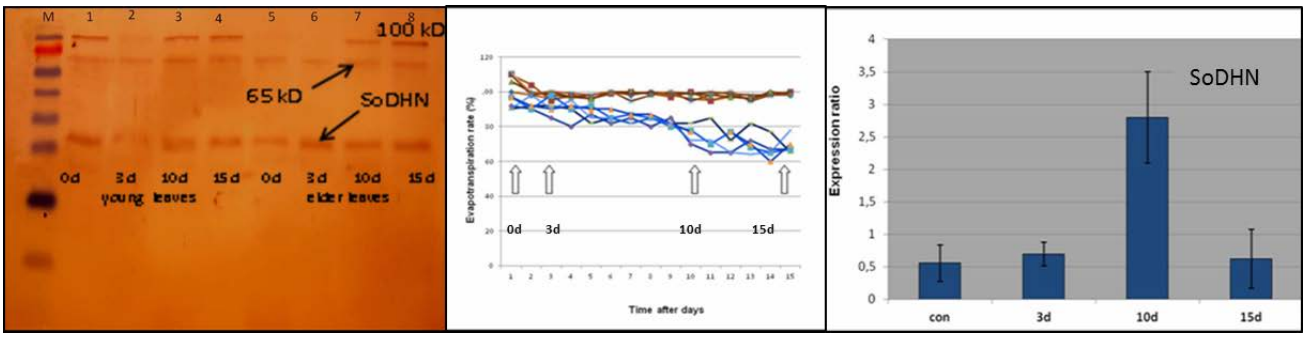


Figure 38: Comparison between dehydrin expression at mRNA (right) and protein level (left) according to the rate of evapotranspiration (middle).

Down-regulation of the SoDHN during ongoing stress

As mentioned above, after an initial increase, the concentration of SoDHN RNA rapidly decreased (Figure 38) during the course of the experiment, although the stress situation was maintained, ascertained by the unchanged low rate of evapotranspiration (Figure 38). In principle there are two explanations for this phenomenon. Either the SoDHN expression is actively reduced or the decline is due to a general reduction in metabolic activity. Such passive decline of the expression level was reported by Kramer *et al*. (2010), who showed that the reduction in dehydrin expression in drying coffee seeds is due to a general shut-down of the entire metabolism. However, in contrast to the dried coffee seeds, which finally revealed a water content of only about 10 %, it is much higher in the leaves of the sage plants subjected to drought

stress. Since they are metabolically full active, such passive decline of the SoDHN expression can be excluded.

In principle, an actively regulated decline in the content of the SoDHN transcript could either be due to a withdraw of an up-regulation or to a deliberate down-regulation by another factor, e.g. a repressor. As the regulation of the multiple processes induced by drought stress is very complex and the various reactions might influence and regulate each other, it is not really possible to distinguish between these both options. Accordingly, in this work the observed decline in SoDHN expression is termed as"down-regulation", albeit the actual reason is not elucidated yet. In this context, it should be noted that apart from signal transduction pathways involving or lacking abscisic acid, a complex regulation networks are involved in the regulation of the metabolic syndrome of drought stress metabolism (Chaves *et al.*, 2003; Shinozaki and Yamaguchi-Shinozaki, 2006).

Yet, the evaluation of the relevance of up- and down-regulation of dehydrins is much more sophisticated, since the expression of dehydrins is not only impacted by drought but also by other factors, e.g. cold or salinity. Corresponding results have been reported by Nylander *et al.* (2001), who estimated the expression of five dehydrins (COR47, LTI29, ERD14, LTI30 and RAB18) in *Arabidopsis thaliana*. Whereas ERD14 accumulated already in unstressed control plants, expression of ERD14 was upregulated by salinity and low temperature. Similar findings also were reported by Olave-Concha *et al.* (2004) who analysed seven dehydrins (58, 57, 55, 53, 48, 30 and 27 kDa) in the leaves of *Deschampsia antarctica.* The authors showed that different treatments like NaCl, PEG, low temperature and exogenous ABA resulted in different pattern of the accumulation of the dehydrin proteins. Dehydrin 58 kDa was expressed in all treatments, but it was more enhanced by exogenous ABA as by PEG treatment. In contrast dehydrin of 30 and 27 kDa were accumulated at 24 h of ABA treatment and slightly detected under salt stress. These examples and coherences vividly demonstrate the requirement for further research in this complex scientific field.

Constitutively expressed dehydrins

As shown in the previous chapters, SoDHN is constitutively expressed at a low level, but it is up-regulated under drought stress. The related dehydrin protein is stable and is present at an enhanced level during the entire stress situation. Accordingly, it could be assumed that the

significance of SoDHN might be based on various functions, i.e. to enable or maintain plant's metabolism under normal conditions or to protect the cell against the impact of stress. This is in agreement with most other findings on dehydrin expression in other plants. In *Arabidopsis thaliana*, about 10 dehydrins are described (Hara, 2011). Some of them are up-regulated, e.g. LTI30 (Nylander *et al.,* 2001) and some are expressed constitutively, e.g. AtHIRD11 (Hara *et al*., 2011). Based on the finding that both, the transcription level of AtHIRD11, and the level of the corresponding dehydrin protein remained constant in *Arabidopsis thaliana* under different stress conditions such as NaCl, cold, mannitol; Suggesting that AtHIRD11 dehydrin might play a role as housekeeping gene. In contrast, LTI30 seem to be important for the withstanding of certain stress situation. Unfortunately, no clear picture on the specific functions of the dehydrins could be drawn.

Conclusions

One major goal of this thesis was to answer the question whether or not the expression of dehydrins could be used as a reliable stress marker as proposed by Hanin *et al*. (2011). In this context, SoDHN was chosen as suitable candidate for sage plants. The studies clearly outlined that a quantification of the changes in the expression level due to stress are not sufficient to estimate the potential stress level. The main reason for this is the down-regulation of SoDHN although the stress situation is maintained. However, the SoDHN protein remains at an enhanced level all over the time the stress situation lasts. Obviously, the SoDHN protein is involved in the protection of the cell against the consequences of the stress applied, yet, due to the stability of the protein only a very short phase of enhanced expression is required. As dehydrins in general are described to be very stable, corresponding discrepancies between the level of gene expression and the protein level will arise also in other plants. Indeed, there might be some dehydrins, which do not follow this pattern and whose proteins will be removed shortly after their biosynthesis. In consequence, expression and protein content will match and thus might be suitable candidates for the required stress marker. However as long sound information is lacking about the entire issue, apart from the expression studies, always supplemental Western blot analyses have to be applied. This approach also will enable the identification of those dehydrins which putatively represent some kind of housekeeping genes. Accordingly, only a combination of gene expression and protein quantification could be used to characterize the actual stress status.

4.5. Impact of drought stress on monoterpene content

In order to elucidate the general impact of drought stress on secondary metabolism, in this investigation, sage was used as model system. Accordingly, apart from the drought stress induced changes in the contents of monoterpenes also the corresponding stress situations have to be monitored. Thus, in parallel to the estimation of monoterpenes, also the expression of SoDHN and the related protein contents had been estimated and used - as mentioned above - as suitable system to monitor the actual stress status. In order to estimate whether or not the observed changes in monoterpene content are solely due to changes in the over-reduced states, as outlined in the introduction, or if they are also could be due to changes in the biosynthetic capability, in addition to the quantification of monoterpenes also the expression of the key enzymes in monoterpene synthesis, i.e. the monoterpene synthases, had been monitored.

4.5.1. Drought stress induced changes in monoterpene content

As mentioned, drought stress was applied to the sage plants by gradually reducing the soil water content by reducing the amount of daily water until the evapotranspiration rate decreased down to about 70 %, where it was maintained for three weeks (Figure 30). Sampling was performed in parallel to that for dehydrin analyses, i.e. after 3 days, when the evapotranspiration started to decrease, after 10 days, when the rate reached the required 70%, and after 15 days, when the drought stress was kept for nearly one week. Moreover, control samples had been taken at the beginning of the experiment. Monoterpenes had been quantified by gas liquid chromatography (GLC).

The corresponding GLC analyses revealed that the main constituents of essential oils are α- and β-thujone, followed by camphor, and 1,8-cineole, respectively (Figure 9). Whereas the accumulation of α- and β-thujone increased gradually in the course of the experiment and is doubled after 15 days (Figure 39A). In contrast, the concentration of 1,8-cineole first increased and then slightly decreased again. The related changes in the concentration of camphor is in-between; after a initial increase, it decreases again; however, highest concentration was detectable after 15 days, where the concentrations was about six fold higher than at the start (Figure 39A).

To facilitate the interpretation of these results with respect to the central question of this thesis, the concentrations of the single monoterpene were combined and displayed in Figure 39 B

as total monoterpenes concentration. These results clearly mention that the concentration of monoterpenes increased significantly when the leaves were suffering drought stres.

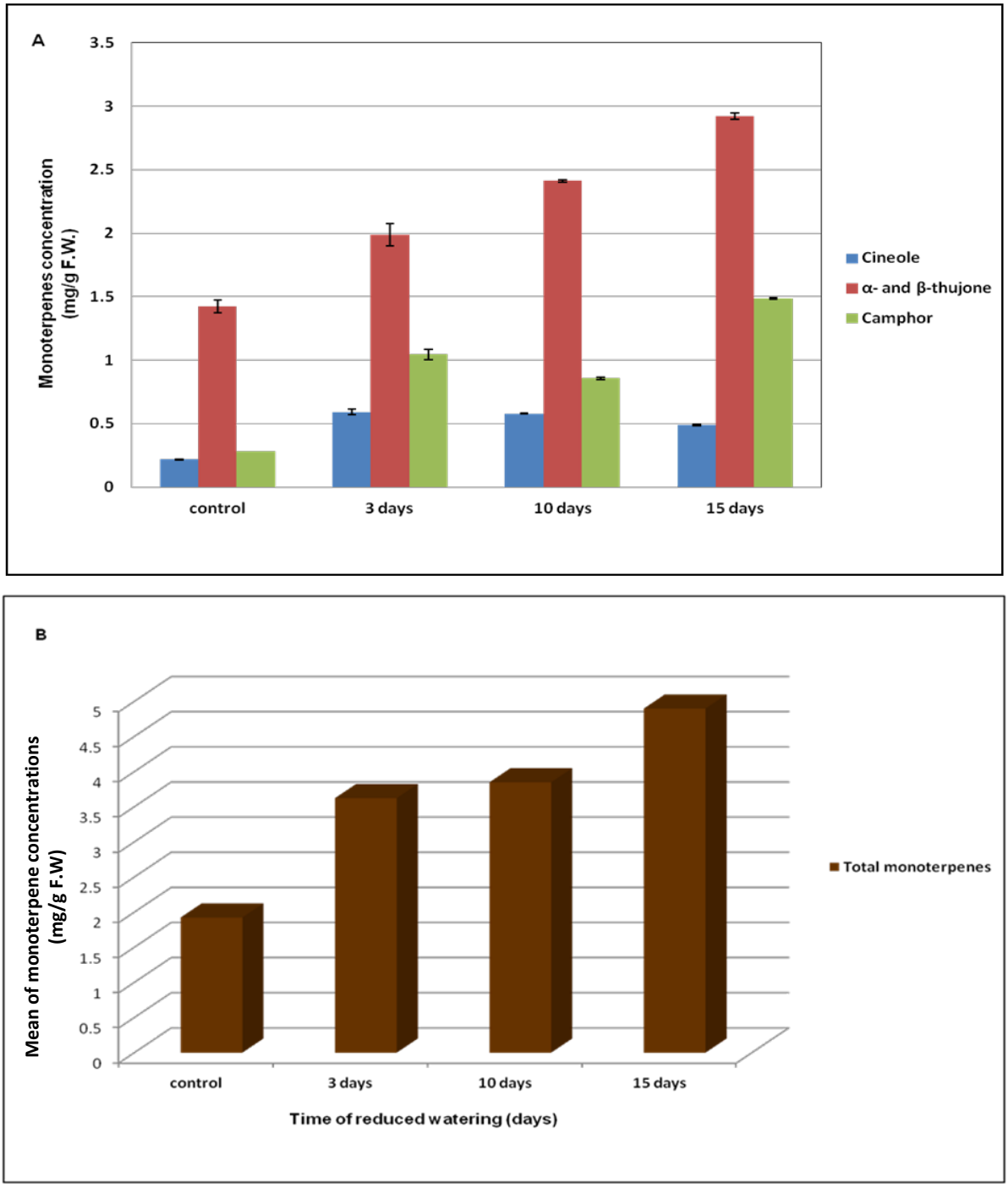


Figure 39: Effect of drought on monoterpene accumulation in *Salvia officinalis*. A: the concentrations of1,8-cineole, α- and β-thujone and camphor in young leaves, B: Mean of monoterpene concentration. Sampling time by days. Values represent the mean of 2 independent experiments. Error bars represent ± standard deviation

In this context, it has to be considered that this increase must not unequivocally result from a real enhancement of monoterpene biosynthesis, but could also be due to the drought related reduction in growth while the biosynthesis remains constant. i.e. the enhancement of monoterpene concentration just reflects the reduction in biomass production in response to drought stress and not an increase in the rate of biosynthesis (Selmar and Kleinwächter, 2013). However, in the present approach, the vegetative growth of both trials had been very similar; accordingly, no significant differences in the biomass of the well-watered plants and those submitted to drought stress had been occurred. Consequently, a reduction in the biomass gain combined with an unchanged rate of biosynthesis, and thus yielding in a higher concentration of natural products in drought stressed plants could be excluded, as it is true for various papers (for review see Selmar and Kleinwächter, 2013). This is confirmed by the findings of Nowak *et al.* (2010), who also showed that drought stress strongly increases the concentration of monoterpenes in sage. In addition, these authors estimated the entire content of monoterpenes per plant (monoterpene concentration x total fresh weight per plant) and determined a significant increase of the total amounts of monoterpenes per plant, too. Consequently, the rate of monoterpene biosynthesis truly is enhanced in drought stressed sage plants compared to that of well watered ones.

Similar previous few studies determined the content of secondary metabolites reported on other plant species cultivated in the different stress conditions. For instance, De Abreu and Mazzafera (2005) compared between the total amounts of phenolics in *Hypericum brasiliense* in drought stressed and control plants. The authors showed that the total content of the phenolic compounds is drastically higher in plants grown under drought stress than in the control plants. Contrary to this study by Manukyan (2011) who mentioned that the total content of terpenoids in *Melissa officinalis*, *Nepeta cataria* and *Salvia officinalis* is decreased, although their concentration was increased.

Yet, such increase in biosynthesis could be based on two different facts; either the increase is caused by the strongly enhanced stress related over-reduction by promoting all reactions consuming $NADPH + H^+$ as mentioned in the introduction, or it is due to an increase in the activity of the enzymes involved. In order differentiate between these both options, the expression of the key enzymes in monoterpene biosynthesis, the monoterpene synthases was estimated.

4.5.2. Changes in the expression of monoterpene synthases in detached leaves

As mentioned above, GLC-analysis revealed that the three most predominant cyclic monoterpenes in *Salvia officinalis* are cineole, camphor and α / β -thujone which account for more than 95% of the total monoterpene amount (Nowak *et al*., 2010). Accordingly, this study focuses on the regulation of the biosynthesis of cineole, camphor and α / β–thujone. The key enzymes for the biosynthesis of these monoterpenes are the cineole synthase for cineole, (+)-bornyl diphosphate synthase, which provides the precursor of camphor, and the sabinene synthase, generating sabinene, the precursor of thujone (Figure 40).

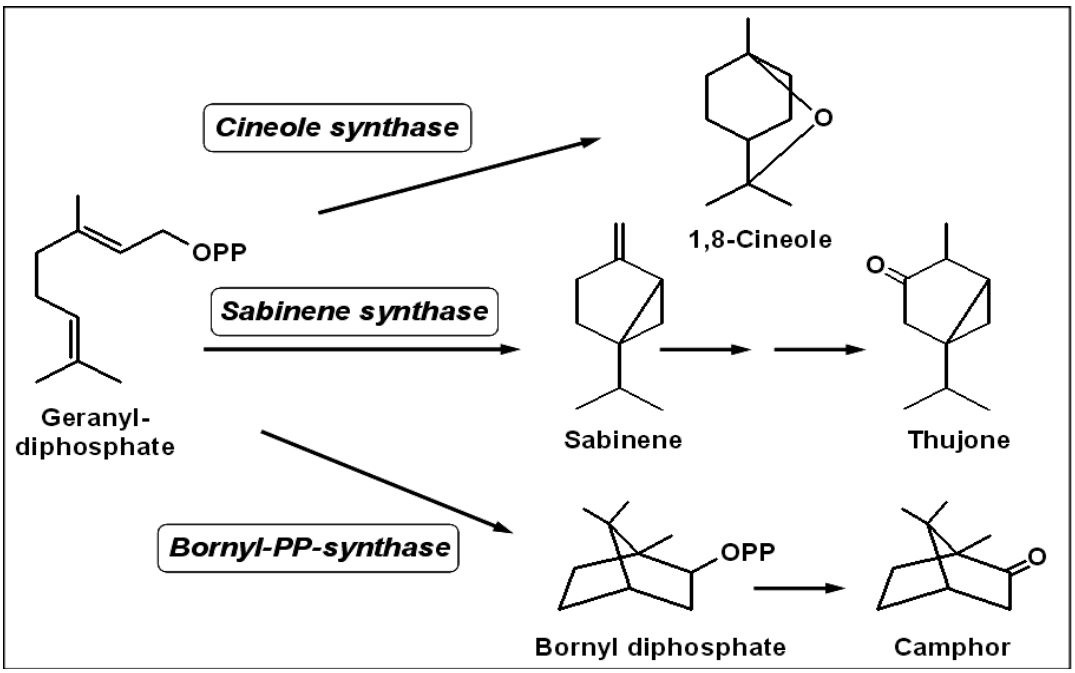


Figure 40: Biosynthesis of the main monoterpenes in sage (Selmar and Kleinwächter, 2013).

Based on the known sequences of the corresponding monoterpene synthases (Wise *et al*., 1998), appropriate primers had been designed (see III.1.7.6) in order to analyze putative stress induced changes in gene expression of these enzymes using Real Time-PCR. As mentioned for the SoDHN, also for monitoring the expression of monoterpene synthases, the system was optimized using detached middle-aged sage leaves to mimic drought stress.

In middle-aged leaves, (+)-bornyl diphosphate synthase is expressed constitutively, since the related m-RNA already is present in unstressed leaves. However, it is significantly up-regulated by drought stress. Already 2 hours after detaching the leaves (Figure 40), the expression level

nearly is doubled. Expression gradually increased further on, until the maximum level is reached at 6h (corresponding to a relative amount of water of about 52 %). Subsequently, when the relative water content declined further, the relative expression of (+)-bornyl diphosphate synthase sharply decreased. In principle, this decline could be caused by a down-regulation. However, it is much more likely that it is due to a general shut-down of metabolism due to the lack of water, inasmuch as the water content at 12 and 24 hours, was only about 42 and 34 %, respectively.

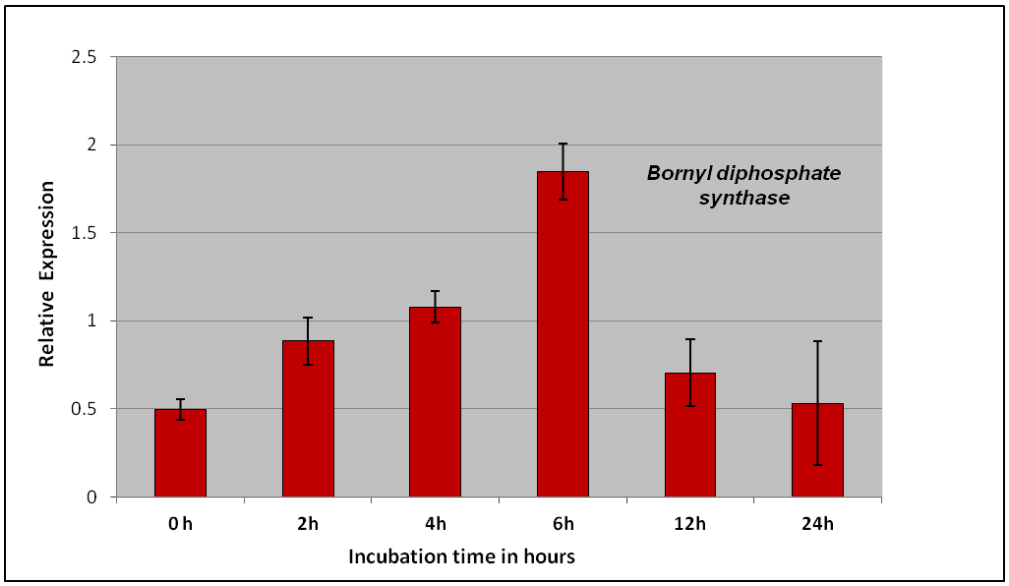


Figure 41: Expression of bornyl diphosphate synthase in detached middle-aged sage leaves. Relative gene expression was quantified by Real Time PCR at various hours after detaching the leaves. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments.

In contrast to the relatively high expression level of bornyl diphosphate synthase, nearly no expression of cineole synthase is present in unstressed leaves. However, cineole synthase strongly is up-regulated in response to water shortage. Maximum expression level is reached after 6 hours (Figure 42). Analogue to the expression of bornyl diphosphate synthase, the expression of cineole synthase also declined when the leaves are dried further on, putatively due to the metabolic shut down due to the extreme the water shortage.

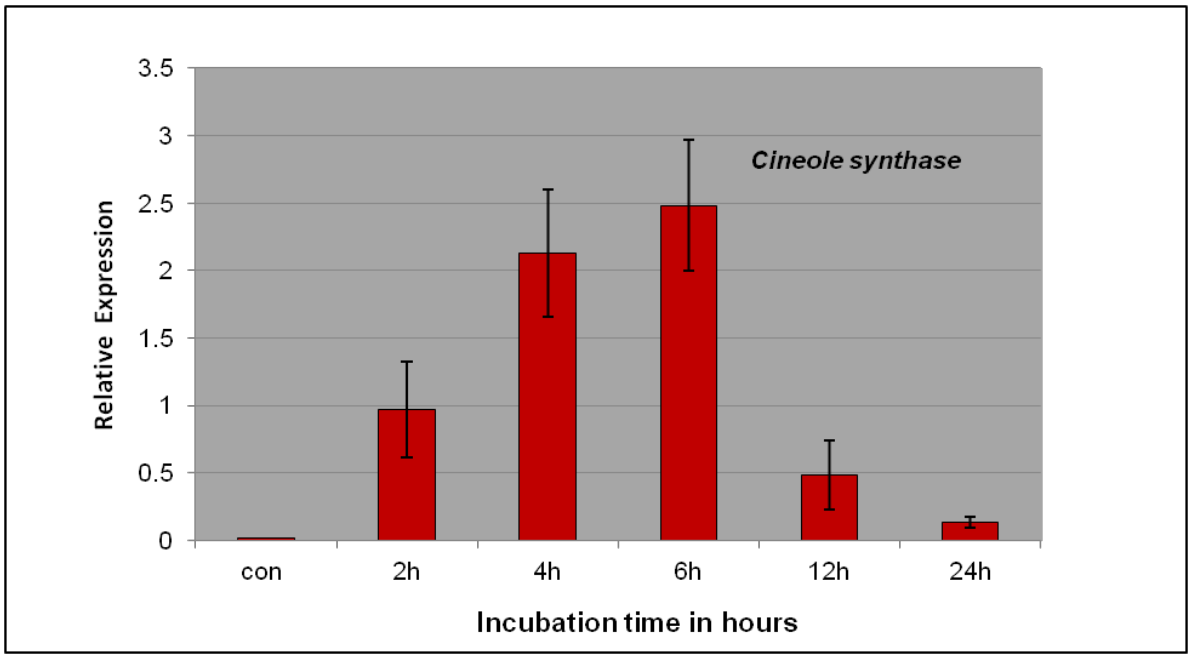


Figure 42: Expression of cineole synthase in detached middle-aged sage leaves. Relative gene expression was quantified by Real Time PCR at various hours after detaching the leaves. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments.

As determined for the bornyl diphosphate synthase, also sabinene synthase reveals a relatively high expression level in unstressed leaves. Yet, drought induced its up-regulation significantly. Highest expression level was detectable at 4 h after detaching, corresponding to a relative amount of water of about 68 %. Later on, in the same manner as determined for the both other synthases, also the expression of sabinene synthase massively decreases, putatively due to the metabolic shut down caused by the extreme water shortage.

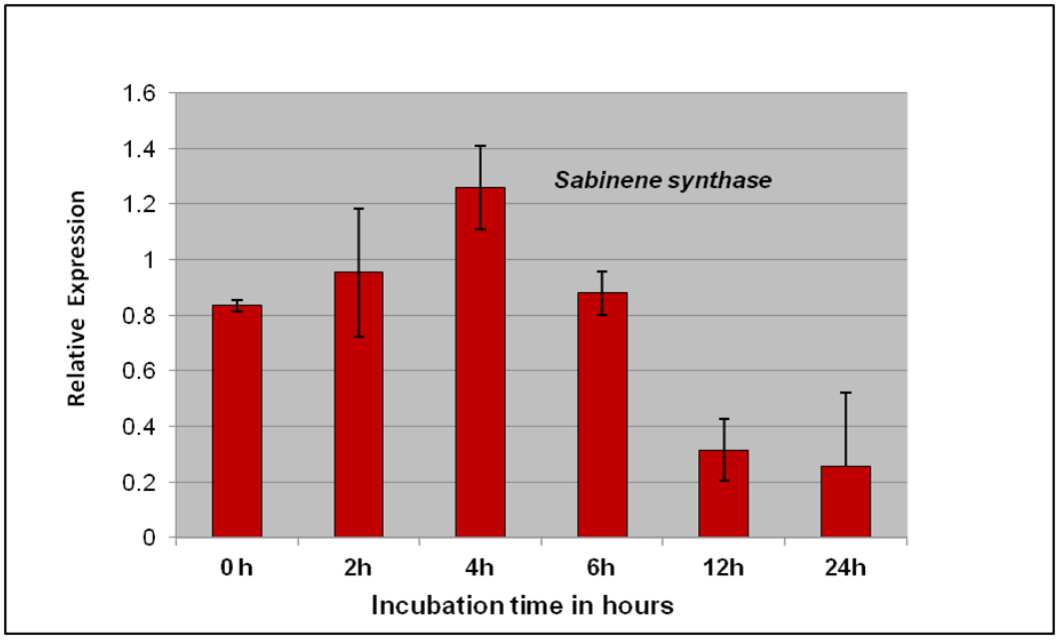


Figure 43: Expression of sabinene synthase in detached middle-aged sage leaves. Relative gene expression was quantified by Real Time PCR at various hours after detaching the leaves. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments.

The results on the up-regulation of monoterpene synthases are surprising. Obviously the drought stress induced enhancement of monoterpene biosynthesis mentioned above could also be due to an increase in biosynthetic capacity rather than being exclusively due to the stress related over-reduction. Yet, for more specific conclusion, further investigations using intact sage plants submitted to drought are required, in order to exclude that the observed expression of monoterpene synthases not induced - at least in part - by the wounding due to the detaching of the leaves. Nevertheless, these results clearly demonstrate that the applied approach represents a reliable procedure to monitor drought stress induced changes in gene expression of monoterpene synthases.

4.5.3. Expression of monoterpene synthases in young leaves of drought stressed sage plants

As mentioned before, in order to elucidate the overall impact of drought stress on the biosynthesis of monoterpenes in sage, the corresponding expression studies for the key enzymes of monoterpene biosynthesis have to be performed using sage plants grown under natural conditions. Drought stress had been applied as outlined above in the context of monitoring the drought induced expression of SoDHN and sampling was the same, i.e. 3, 10, and 15 days after the start of the experiment. The concomitant monitoring of SoDHN expression and that of monoterpene synthases should allow to elaborate further insights how stress impacts on secondary metabolism.

Transcript levels of terpene synthases in young sage leaves of the first node are higher than those of the third node, representing middle-aged leaves, however, and no expression was detected in leaves of node five, being already fully mature (Schmiderer *et al*., 2010). Obviously, the expression of monoterpene synthases in sage strongly depends on the development. Accordingly, in order to rule out any interference with changes in the expression level due to developmental processes, in this study, only young and middle aged leaves from the first and second node had been used. However, in this context, it has to be mentioned that in other species different coherences between leaf age and monoterpene expression have been reported. In peppermint (*Mentha_x piperita*), maximum rate of monoterpene biosynthesis was detected in 15 day old leaves, whereas in leaves and also in very old ones (more than 20 days) far lower rates had been determined (Gershenzon *et al*., 2000).

In contrast to the very similar expression patterns of monoterpene synthases in detached leaves, more heterogeneous expression levels have been observed when analyzing the expression patterns in leaves of intact sage plants submitted to drought stress.

In response to drought, expression of sabinene synthase increased gradually, reaching about twice the initial level after 15 days (Figure 44 A). This is in accordance with the results obtained from the detached leaves, i.e. sabinene syhthase is constitutively expressed to a certain level and markedly up-regulated by water shortage. This performance nicely matches with the changes of the related monoterpene synthesized by this enzyme, i.e., thujone (Figure 44 B).

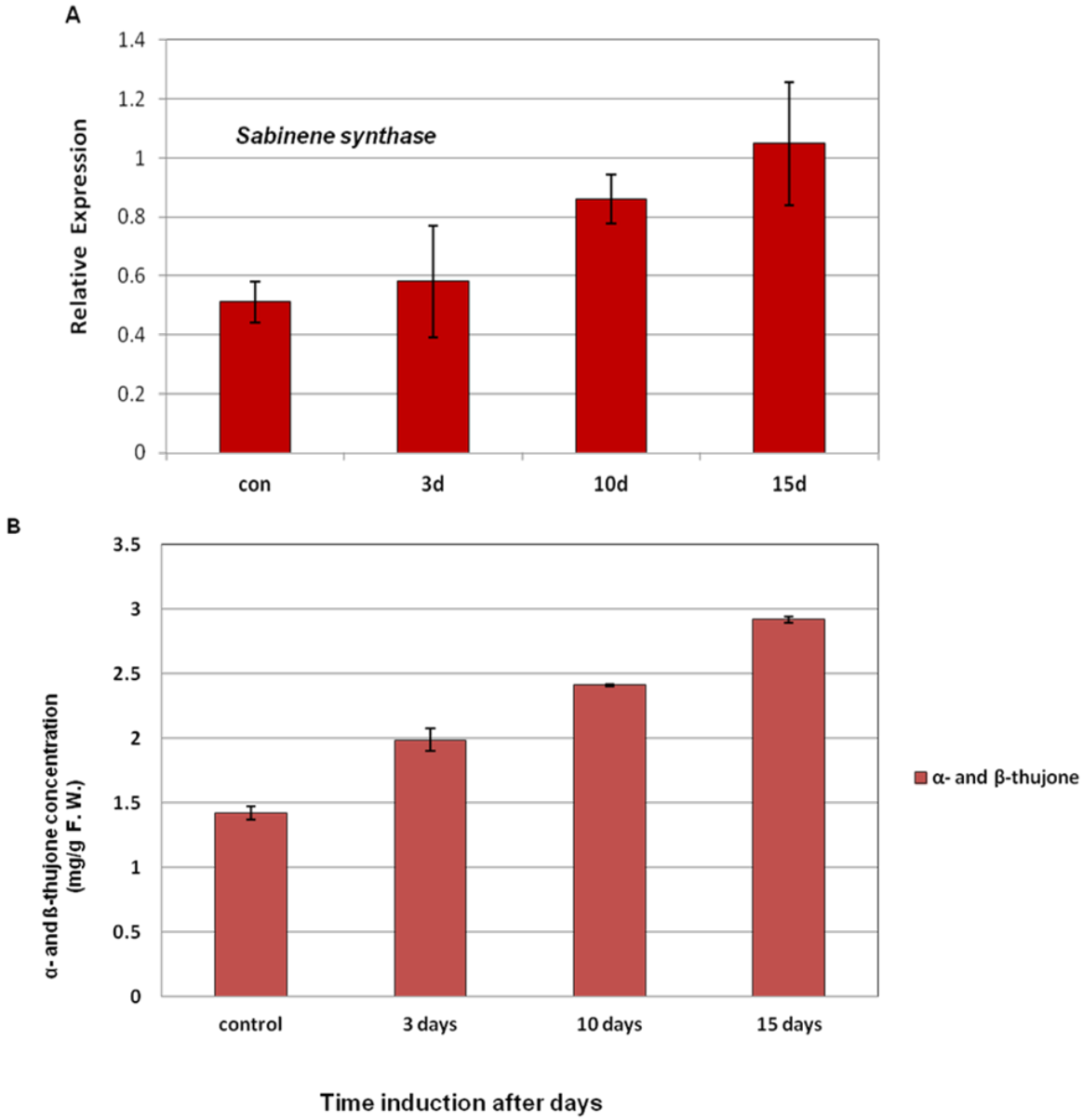


Figure 44: Comparison of sabinene synthase relative expression (A) and concentration of α / β -thujone in young sage leaves of intact sage plants submitted to drought stress. Relative gene expression was quantified by Real Time PCR. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments.

The expression of bornyl diphosphate synthase also is strongly up-regulated in response to drought stress (Figure 45 A). However, in the same manner as shown for the SoDHN, the concentration of the corresponding m-RNA decreases again, resulting in a maximum expression level at day 3, i.e. the moment when the evapotranspiration in the drought stress started to decrease. Thereafter the transcript level continuously declined.

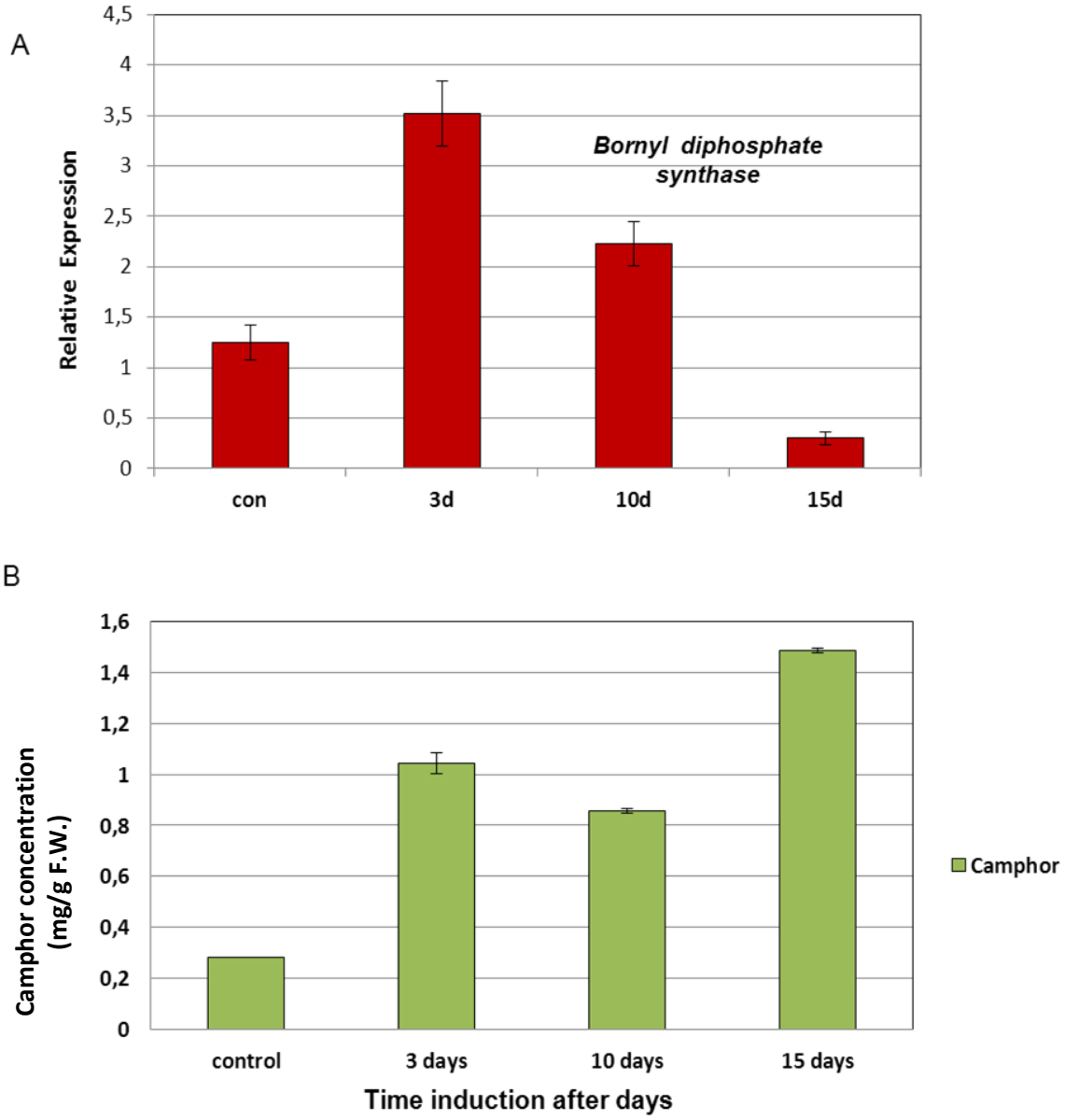


Figure 45: Comparison of bornyl diphosphate synthase relative expression (A) and concentration of camphor in young sage leaves of intact sage plants submitted to drought stress. Relative gene expression was quantified by Real Time PCR. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of 2 independent experiments

In comparison to the corresponding expression profile in detached leaves, that of intact plants is slightly different. A possible explanation for this could be based on the fact that in detached leaves the water content steadily decreased further on, whereas it remained constant when the aspired stress level was reached. The observed stress related expression pattern could be exemplified, if we assume that – as outlined for the transient SoDHN expression – similarly also the bornyl diphosphate synthase might be triggered by the extent of the changes in water content rather than by its actual level. In the case, it could be speculated that the corresponding protein might be relatively stable in order to maintain its activity throughout the entire stress situation. Indeed, the data on the camphor accumulation support this assumption. In the last week of the experiment, camphor concentration markedly increased although the content of m-RNA encoding the bornyl synthase drastically decreased. This means that at this period still sufficient active enzyme protein must have been present.

Whereas the drought stress related expression pattern of bornyl diphosphate synthase and sabinene synthase in principle are similar for detached leaves and for intact plants – both are mainly up-regulated – those of cineole synthase strongly differs (Figure 46 A). This accounts for both, the basic level as well as the progression. In contrast to the previous experiment with detached leaves, where cineole synthase expression was nearly lacking, in the actual experiment with intact plants, this enzyme is strongly expressed already in the control plants. In principle, there are two explanations for this contradictory finding; either the age of the leaves had not been completely [4] identical, or the plants used in the second experiment already had been stressed, e.g. by pathogen attack[5].

[4] Schmiderer et al., (2010) demonstrated that transcript levels of terpene synthases in young sage leaves of the first node are higher than those of the third node (representing middle-aged leaves). No expression was detected in leaves of node five (representing fully mature leaves). Thus, expression of monoterpene synthases in sage strongly depends on leaf development and therefore on the actual age of the leaf.

[5] Although massive efforts had been made to exclude any herbivores and pathogens from the cultivation chamber, some minor invasions could not have been excluded entirely. Since such biologic stress situations also could influence the expression of enzymes involved in secondary metabolism, plants might be affected and consequently might have reacted differently.

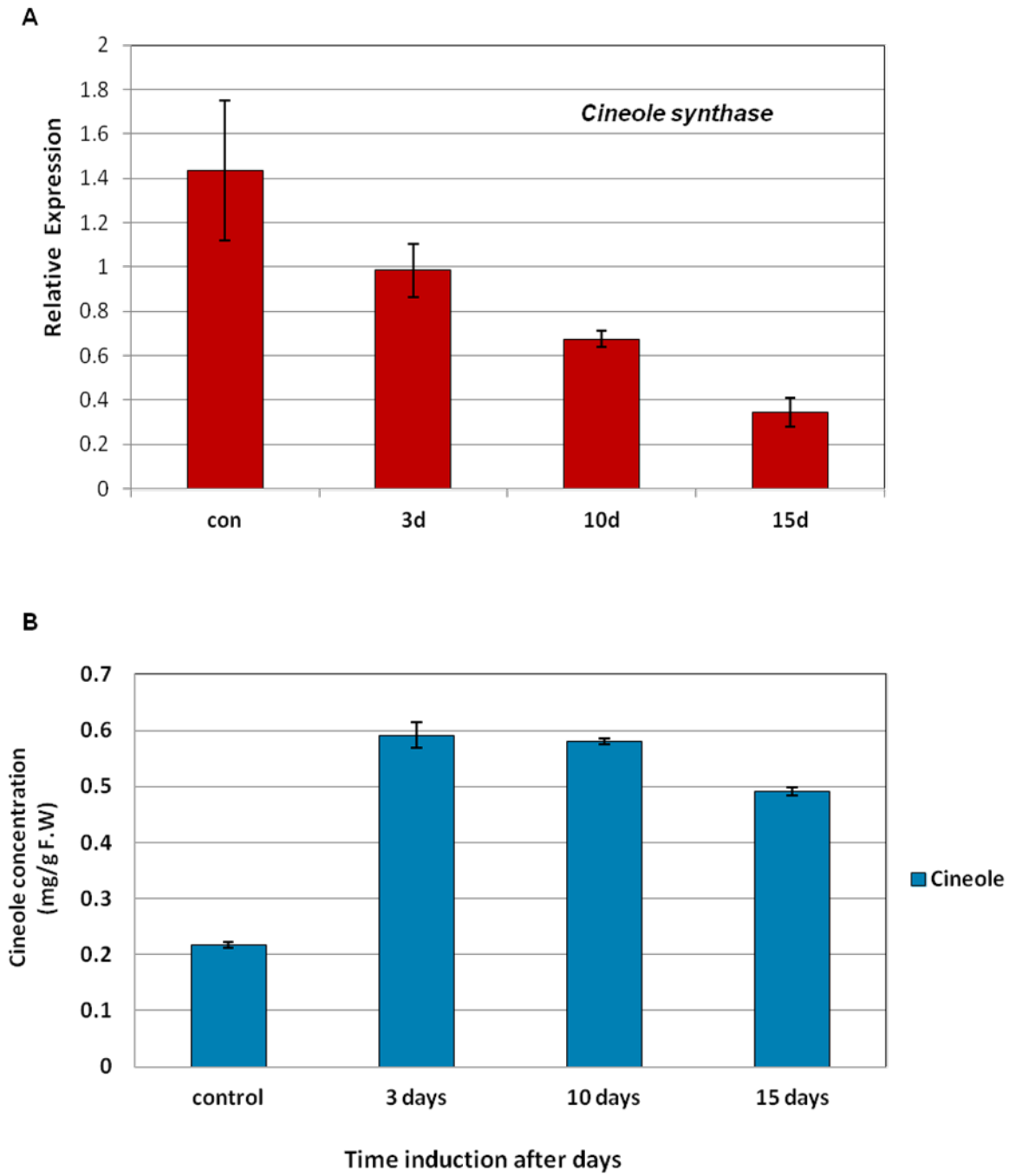


Figure 46: Comparison of cineole synthase relative expression (A) and concentration of cineole in young sage leaves of intact sage plants submitted to drought stress. Relative gene expression was quantified by Real Time PCR. Each value represents 2 independent experiments, each consists of 3 replicates. Error bars represent the ± SD of independent experiments.

Apart from the massive expression of cineole synthase at the start of the experiment (control), also the subsequent alterations differ significantly between intact plants exposed to drought stress and detached leaves. Whereas in detached leaves a strong drought induced increase was estimated, in the intact plants, the initial high expression level decreased constantly in the course of the experiment. If we assume that the initial level was caused by some exogenous factors (herbivores, pathogens etc.) and this biological stress was gone in the course of the ongoing experiment, the corresponding induction should be withdrawn. In this case, the observed decrease could be the result of an interference of a very strong down-regulation due to the lack of biological stress and an up-regulation as it was observed in the case of detached leaves. That the estimated expression pattern for cineole synthase indeed is sound could be deduced from a comparison with the concentrations of cineole, the product of cineole synthase: due to the down regulation of the cineole synthase, the initially increase of cineol concentration is diminished constantly. However, many further approaches are required to elucidate these coherences.

In this context, we have to consider that the expression of the various monoterpene synthases may differ in their response to various stress factors, i.e. drought stress, biological stress, wounding etc. An overview on this topic has been outlined by Zwenger & Basu (2007), who elucidated many factors, i.e. drought, nutrient, ozone and mechanical stress contributing to the up-regulation of terpene synthesis genes in *Arabidopsis*. In contrast, in *Eucalyptus polybractea* water stress and nutrient stress were not responsible for variation in the terpene content (King *et al*., 2004). In *Daucas carota*, mechanical stress caused decreasing of terpene production (Seljasen et al., 2001), whereas in Norway spruce (*Picea abies* L. Karst) the emission of terpene was not affected by ozone stress (Lindskog and Potter, 1995).

Yet, the evaluation of the influence of over-reduced states on the monoterpene biosynthesis requires a comprehensive observation of their expression changing under drought stress. So far, there is no data on the expression of monoterpene synthases under drought stress are available in sage plant. However Schmiderer *et al*. (2010) studied the impact of exogenously applied plant growth regulators gibberellic acid and daminozide on the expression of monoterpene synthases. The authors reported that the expression of monoterpene synthase improved with increasing levels of gibberellins, and decreased when gibberellin biosynthesis was blocked with daminozide. On the other hand, 1,8-cineole and camphor contents increased, after the addition of increasing levels of gibberellins. However, the accumulation of of α - β -thujone

was blocked by daminozide. Another study by Grausgruber-Gröger *et al*. (2012) examined the seasonal impact on the three main monoterpene synthases at the level of mRNA expression in young and still expanding leaves of field-grown sage plants. They reported that the mRNA expression of all monoterpene synthases was significantly influenced by cultivar and season.

In other species like mangrove plants it was found that the expression level of multifunctional triterpene synthase increased with salt stress in the roots and leaves, suggesting that the terpenoid may be involved in the protection of mangrove from salt stress (Basyuni *et al*., 2009) Also, Zwenger and Basu (2007) demonstrated a positive correlation between terpens transcription against salinity in *Arabidopsis thaliana.* Williams (1999) mentioned that triterpenoids showed positive insecticidal activity in *Rhizophora mangle* (Rhizophoraceae).

Taken together, it seems that many factors contribute in the induction of monoterpene synthases in particular and terpenes synthases in general. Furthermore the expression of the different monoterpene synthases is regulated differentially.

4.6. Impact of drought stress on secondary metabolism

The major goal of this work was to gain more information on the drought stress related impact on the accumulation of monoterpenes in sage for further elucidating the complex interactions between stress and secondary metabolism. Accordingly, based on the knowledge that in drought stressed sage plants monoterpene concentrations are enhanced (Nowak *et al.*, 2010), comprehensive investigation on both, the stress status and the biosynthesis of monoterpenes had been elaborated, in order to verify the hypothesis that the stress related enhancement of monoterpene concentration is caused by the over-reduced states occurring in stressed leaves.

4.6.1 Stress related impact on monoterpene synthases

According to the basic concept on the interaction of stress and secondary metabolism outlined in the introduction, it was assumed that any increase of natural product biosynthesis solely is related to the enhancement of all reactions consuming $NADPH + H^+$. However, the expression analyses revealed that monoterpene synthases – apart from some exceptions - overall strongly are up-regulated. If we assume that this up-regulation enhances the related enzyme content, we have to state that in drought stressed sage plants the biosynthetic capability for monoterpene synthesis is intensified, too. This however, means that the observed increase in

monoterpene content – at least in part – also resulted from up-regulated enzyme activities rather than only from a shift by an enhanced level of $NADPH+H^+$. Thus, the question on the underlying biological relevance arises. Indeed, monoterpenes – like other natural products - are known to be important factors within the various interaction of the plants with their environment (for review see Hartmann, 2004 & 2007): Due to their repelling effect, they protect plants against potential herbivores, their toxic properties are the bases for the defence of pathogens, and, due to their characteristic scents and odors, they represent important factors for attracting potential pollinators (Van Poecke *et al*., 2001; Heil and Bueno, 2007). Based on these insights, there is no sound explanation, why – under a certain drought stress situation, in which none of these functions are altered – in the course of evolution, an up-regulation of monoterpene biosynthesis should have been developed. Accordingly, we have to admit that – in addition to the well-known ecological functions – the overall relevance of monoterpene biosynthesis should be complemented by an additional relevance, i.e. a function, which is related to the metabolic situation in drought stressed plants.

In this context we have to consider that their synthesis *per se* is thought to be a further mechanism for dissipating the surplus of energy, which in general is absorbed by plants in the natural environment (Wilhelm & Selmar, 2011). An intriguing example for this assumption is the strong isoprene emission from numerous plants. Several authors postulated that the energy and reduction equivalents required for the biosynthesis of isoprene emitted by leaves, indeed could significantly contribute to the dissipation of the excess of photosynthetic energy (e.g. Sharkey and Yeh, 2001; Fall 1999). An assessment of the energy used for photosynthesis in comparison to that consumed for isoprene synthesis under standard conditions revealed that the energy consumption accounts for less than 1% (Magel *et al.*, 2006). However, under stress situations, i.e., at higher temperatures, the amount of energy dissipated by isoprene emission strongly increases and could account up to 25 % of net photosynthesis energy supply (Magel *et al*., 2006). Accordingly, at least in some cases, the strongly enhanced biosynthesis of terpenoids represents an additional tool to dissipate the surplus of energy and thus contributing to the decrease of the amount of oxygen radicals produced under stress.

If indeed the stress related increase in natural product biosynthesis contributes significantly to energy dissipation, this should have implications for the general understanding of the evolution of natural products. There is no doubt that the basic and general statements of

ecological biochemistry are correct and appropriate. However, if the production of highly reduced substances *per se* may reveal an evolutionary advantage - even without any direct ecological significance, their biosynthesis could be maintained in the course of evolution, i.e. due to a positive selection of the corresponding plants by being "better protected" against an over-supply of energy. Accordingly, in the course of evolution, several steps of the biosynthesis of a certain compound or intermediates could be maintained, even before the compounds reveal any ecological effect, as repellent, attractant, etc. In consequence, the selection and manifestation for biosynthetic pathways comprising numerous steps and yielding in final products obtaining high ecological effects should be much more facilitated. Even when the precursor substances, which intermediary are generated, reveal no ecological effect, their selection could be favoured due to the additional option of energy dissipation in consequence of their biosynthesis.

4.6.2. Consequences of the stress related over-reduced states

As outlined above, due to the drought stress related up-regulation of monoterpene synthases, the drought stress induced increase in monoterpenes could be – at least in part – also be caused by the enhanced corresponding biosynthetic capacity. However, based on the concept that the enhanced biosynthesis of terpenoids represents an additional tool to dissipate the stress related surplus of energy, we have to assume that in stress situations the over-reduced status indeed is raised. This assumption vividly is confirmed by the fact that under stress conditions an "overflow" of electrons from the photosyhthetic electron transport chains, denoted as Mehler reaction, occurs. The generation of the related O_2^- radicals is expounded by the stress related up-regulation of superoxide dismutase and ascorbate peroxidase (see Figure 6), responsible for the detoxification of the corresponding radicals. Obviously, despite of the various protective mechanisms dissipating the surplus of photon energy, i.e. non-photochemical quenching, photorespiration and the xanthophyll cycle (Asada, 2000; Alscher *et al*., 2002; Chen *et al*., 2004; Pitzschke *et al*., 2006), the over-reduced status is raised. The resulting increased content of reduced $NADPH+H^+$ should shift all reaction equilibria towards the production of highly reduced compounds. Accordingly, also the monoterpene synthesis should be increased "passively" by the stress related over-reduced status.

The results outlined in this thesis reveal a high significance for the strategy of further approaches to elucidate the stress related impact on secondary metabolism as well as for the

general understanding of this issue. With respect to forthcoming experimental works, it is absolutely necessary to quantify the total amounts of natural products rather than only their concentrations. Only a comparison of the entire contents before and after the related elicitation could attest whether or not there had been an increase in biosynthesis. In the past, it was assumed that such increase exclusively is caused by the shift of the redox state. However, based on the results presented here, we have to distinguish between the "passive" increase of biosynthesis due to a shift in redox state and an "active" increase due to the enhanced biosynthetic capacity resulting from an up-regulation of the enzymes involved. Accordingly, in all future works on this issue, apart from the increase of the contents of secondary plant product, also the stress related impact on the biosynthetic capacity has to be determined.

In conclusion, it could be stated that in drought stress situations, due to over-reduced states, apart from the well-known mechanisms for energy dissipation also an increase of the contents of secondary plant products may contribute to prevent a further raise of the stress related generation of reactive oxygen species. Yet, the major outcome of this work is the finding that this increase in biosynthesis not only is due to a "passive" shift of biosynthesis by the over-reduced status but also is caused by an "active" up-regulation of the enzymes involved in the corresponding biosynthesis. There is a massive need for further research in order to elucidate, to which extent similar processes also are realized in other plants, or for other highly reduced natural products. Correspondingly, the question arises, whether or not these coherences correspond to a general issue.

Summary and future perspectives

This investigation was aimed to contribute to the basic understanding of the complex interactions between the so-called stress metabolism and the secondary metabolism. In this context, the drought stress related impact on monoterpene biosynthesis in sage and the applicability of dehydrins as molecular markers for drought stress had been studied. It could be shown that monoterpene biosynthesis is enhanced under drought stress and that dehydrins indeed represent suitable tools to estimate drought stress. The promising results can be summarized as follows:

A: Dehydrin

A comprehensive evaluation of the applicability of dehydrins as suitable stress markers requires an extensive characterization of these small protective proteins. Indeed, when appropriate antibodies are used for the corresponding investigations on protein level, all different dehydrins occurring in sage are covered. Yet when the up-regulation of transcription shall be investigated, the analysis has to be focussed on one certain dehydrin. In this study, the dehydrin SoDHN was chosen. In detail, the following results had been elaborated:

General molecular characterization of SoDHN

- ✓ The dehydrin gene, denoted as SoDHN, was isolated from common sage (*Salvia officinalis)* for the first time. The cDNA sequence of SoDHN has a total length of 1000 bp with putative open reading frame is 735 bp and was submitted to gene bank (accession number: AEB77936.1).
- ✓ SoDHN exhibits two K-segments (highly conserved lysine-rich motifs of 15 amino acids) and one S-segment (numerous successive serine residues). Thus, it represents a SK_2 type dehydrin.
- ✓ SoDHN exhibits a putative consensus sequence site for protein kinase 2-phosphorylation (S-segment followed by three acidic amino acids, in this particular case, three glutamic acids: SSSSSSS-EEE. Moreover, SoDHN reveals a certain lysine motif (KRKKKK), which is speculated to function as a nuclear targeting signal sequence.
- ✓ The calculated molecular mass of SoDHN is 26.96211 kDa, and its isoelectric point is 5.27. Accordingly, the SoDHN protein belongs to the class of acidic dehydrins.
- ✓ The amino acid revealing the highest abundance is glutamic acid with a frequency of 19.7 % (48 glutamic acids are distributed throughout the amino acid sequence of total 244 amino acids), followed by lysine, proline, glycine, alanine and valine, respectively .
- ✓ High amounts of glutamic acid, lysine, serine, proline, alanine and arginine are reported to promote the disorder of proteins. Corresponding analysis using the computer program *Disordered Predication* indeed revealed that SoDHN is partially unstructured.
- ✓ SoDHN heterologous protein was successfully over-expressed in E. *coli* with apparent molecular mass of around 54 kDa.
- ✓ In order to elucidate the reason for the massive differences in molecular weight of the genuine sage SoDHN and theheterologously expressed protein, further research is required.

SoDHN as marker for drought stress

- ✓ SoDHN is constitutively expressed revealing a basic mRNA-level.
- ✓ Under severe drought stress, i.e. in detached leaves, SoDHN expression is significantly up-regulated. Already 4 hours after detaching the leaves, when the corresponding relative water content was about 66%, m-RNA level was enhanced up to 6.5-fold. However, subsequently, the

expression declined continuously, obviously due to the general metabolic shut-down caused by the massive water loss, since the remaining water content was less than 35% already 12 hours after detaching.

- ✓ In order to induce drought stress in intact sage plants, watering was strongly reduced until the evapotranspiration reached 70 to 80% compared to the control. This rate was maintained throughout the entire experiment. SoDHN expression increased fourfold. Unexpectedly, although evapotranspiration remained at the same low level and plants suffered drought stress, dehydrin expression massively declined.
- ✓ In contrast to the transient increase in mRNA expression, the abundance of the dehydrin protein remained stable throughout the entire period, in which drought stress was maintained, documented by the constant evapotranspiration of 70 to 80% of the control. Western blot analysis revealed that SoDHN protein (27 kD) is already present in non-stressed leaves, which nicely fits to the finding that SoDHN is constitutively expressed to a basic level. Drought stress, however, significantly enhanced the content of SoDHN protein, which remained constant although the corresponding transcription level instantly decreased to the basic level. Obviously, the dehydrin protein is relatively stable.
- ✓ In addition to SoDHN protein, on the basis of the Western blot analyses, two further putative dehydrin proteins had been detected, revealing molecular masses of 100kD and 65 kD, respectively. While the 65 kD dehydrin is constitutively abundant and may act as housekeeping gene, the100 kD dehydrin protein is not present in unstressed mature leaves, but in very young ones. Moreover, in mature leaves, it is accumulated massively in response to drought stress.
- ✓ In conclusion: the transient expression of SoDNH allowed to indicate the initiation of the stress situation. However, the monitoring of the actual and ongoing stress level requires the estimation and comparison of SoDHN at protein level. The additional corresponding consideration of the abundance of the100 kD dehydrin would be advantageous.

B: Drought stress related impact on monoterpene synthesis and accumation

To elucidate the complex interactions between drought stress and the secondary metabolism in general, the biosynthesis and accumulation of monoterpenes had been investigated exemplarily. It was postulated that the stress related over-reduction will lead to an increase of the biosynthesis of highly reduced secondary plant products. Indeed, under drought stress, generally the concentration of such natural products increases, however, a corresponding enhancement of the monoterpene concentration might just reflect the reduction in biomass in response to drought stress and not an increase in the rate of biosynthesis. Due to the fact that in this work such stress related decrement did not occur, it could be unveiled that the observed concentration increase indeed results from a real enhancement of monoterpene biosynthesis. However, this increase might not be related only to a “passive” shift in biosynthesis but may also be due to an enhancement in biosynthetic capacity. Accordingly, also the stress related impact on the expression of monoterpene synthases has been analyzed. In detail, the following results had been elaborated:

- ✓ GC analysis revealed that the accumulation of α / β -thujone increased markedly in leaves in response to drought stress and was doubled within 15 days. 1,8-cineole content first was doubled after at 3 days and then slightly decreased again. In the same manner, also the content of camphor increased drastically and was about 4 fold higher already after 3 days. Highest camphor content was detected after 15 days (6 fold). The total amount of monoterpenes increased significantly to about twofold after three days and threefold after 15 days.

- ✓ Drought stress related impact on monoterpene synthases had been analyzed – analogously to the dehydrins – in detached leaves as well as in plants submitted to drought stress. It turned out that the monoterpene synthases revealed individually different expression patterns in detached leaves and in intact plants.
- ✓ In detached leaves, bornyl diphosphate synthase (producing the precursor of camphor) is expressed already significantly in unstressed leaves. However, it is up-regulated already 2 hours after detaching the leaves. Its expression gradually increased until the maximum level is reached at 6h, subsequently the expression sharply decreased. Cineole synthase is almost not expressed in control plants, but is strongly up-regulated and reached a maximal level after 6 hours, and then it declines strongly. In contrast, the relatively high expression levels for sabinene synthase (producing sabinene, the precursor of α / β -thujone) increaed within the first hours after detaching the leaves, reached a maximal level at 4h and decreased again. As mentioned with respect to the corresponding expression pattern of SoDHN, the strong decline in the second phase of the experiment putatively is due to the general metabolic shut-down of the entire metabolism due to massive loss of water.
- ✓ With respect to the expression pattern of drought stressed intact plants, such general metabolic shut-down of the entire metabolism could be excluded. The expression of monoterpene synthases had been determined in those plants, which also had been used for the GC analysis. The expression of sabinene synthase increased gradually and reached a maximum of 2 fold after two weeks. The expression of bornyl diphosphate synthase strongly was up-regulated (3 fold); maximum level was reached already after 3 days. Thereafter, the transcript level continuously declined. In contrast, transcript level of cineole synthase continuously declined.

Conclusion

Dehydrins are reliable stress markers. However, since their expression at mRNA level and the abundance of the corresponding protein could differ depending on the actual stress situation, both parameters have to be monitored in parallel in order to evaluate reliably the genuine stress status. Yet, further experiments are required in order to elucidate comprehensively the regulation and function of dehydrins. In this context - apart from the general aspects of the metabolic significance of dehydrins - the observed differences between the apparent molecular mass of recombinant SoDHN protein and the genuine one occurring in the sage plants are of special interest. Moreover, special emphasis should be placed on the question, whether the drought stress induced transient dehydrin expression is triggered by a certain leaf water content, or if it is caused by the magnitude of its changes.

The drought stress related increase in monoterpene content is not only due to a “passive” shift of biosynthesis caused by the over-reduced status, but also is related to an “active” up-regulation of monoterpene synthases. This enhancement of biosynthetic capacity may indeed have relevance for the general and basic understanding of the evolution of plant secondary metabolites: apart from their well established ecological significances, the biosynthesis of monoterpenes also might contribute - in addition or in complement - to well-known mechanisms for energy dissipation. Accordingly, even before a certain ecological function of a mutation has evolved and manifested during further evolution, the merely existence of a highly reduced substance might by utile and thus be preserved. However, many further experiments are required in order to elucidate the dynamic adaptations of highly reduced natural products under the ecological selection pressure. In this context, the entire synthetic pathways of certain natural products have to be investigated by focusing on the origin of the metabolites and the related history plant.

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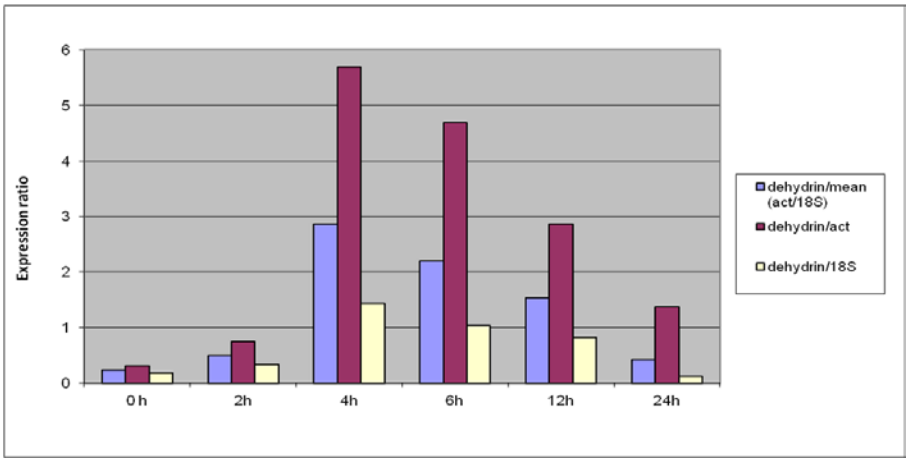
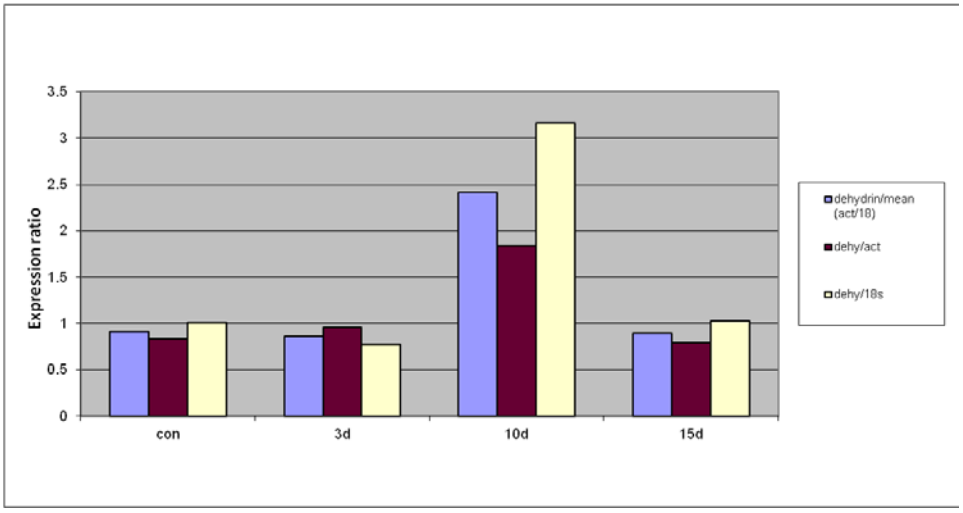
VII. Appendix

- **Quantification of dehdyrin (SoDHN) and monoterpene synthases expression relative to actin and 18 S using Real Time PCR**

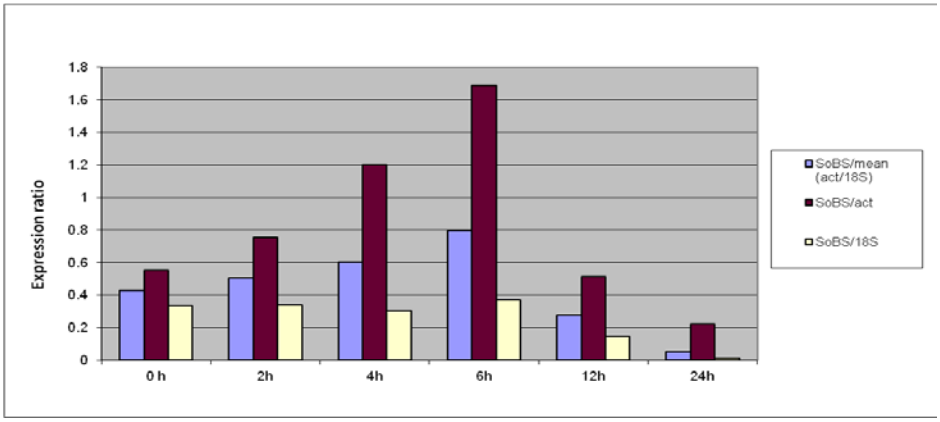
- SoDHN gene expression in detached middle aged leaves (Incubation time by hours)



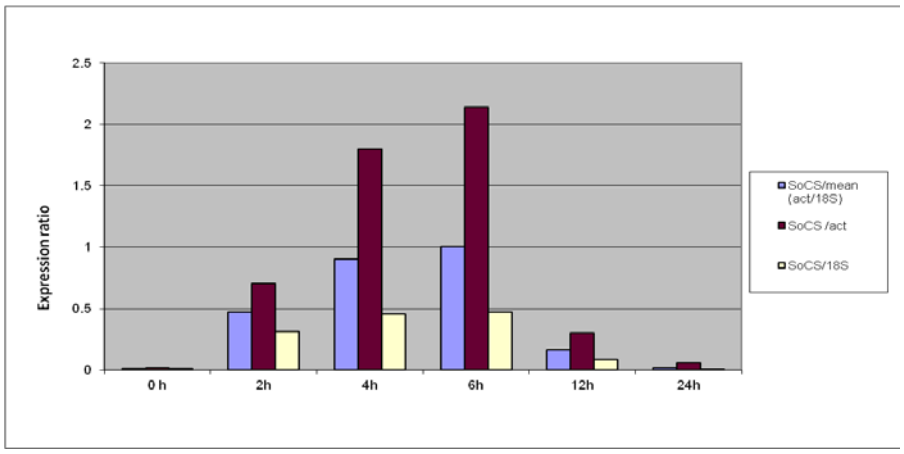
- SoDHN expression profile in intact plants (Incubation time by days)



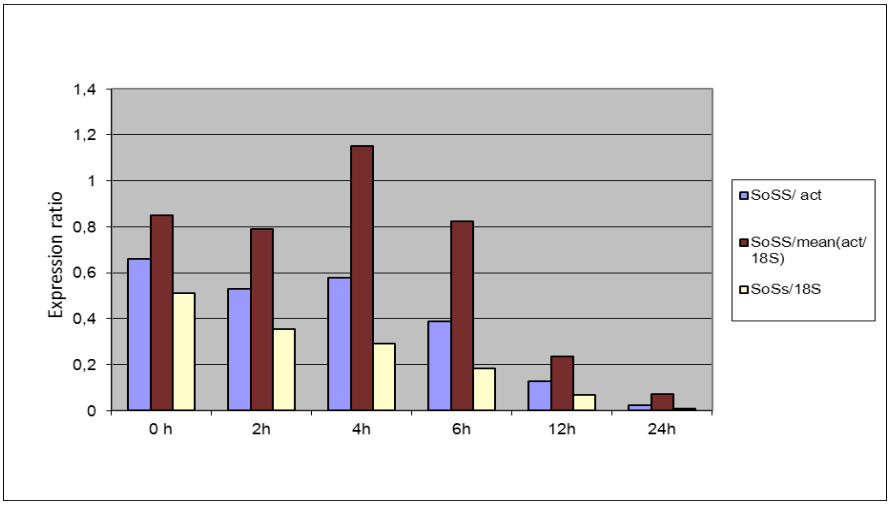
- Expression of bornyl diphosphate synthase (SoBS) in detached middle-aged sage leaves (Incubation time by hours)



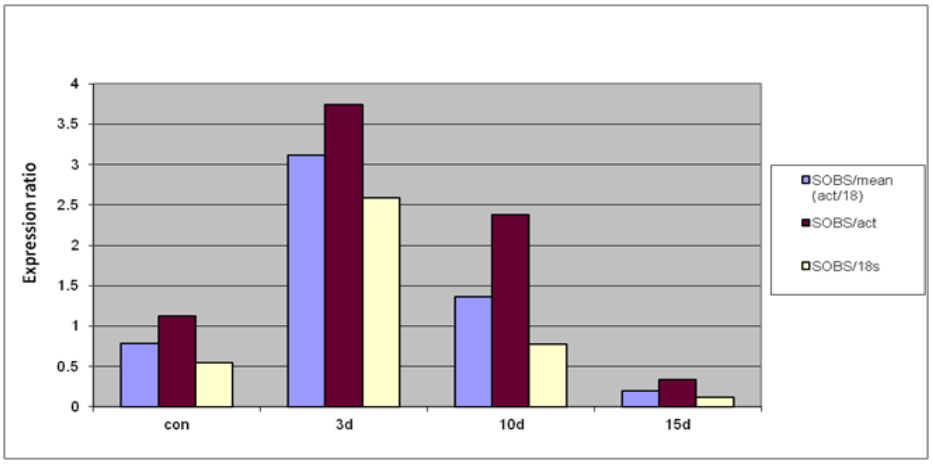
- Expression of cineole synthase (SoCS) in detached middle-aged sage leaves



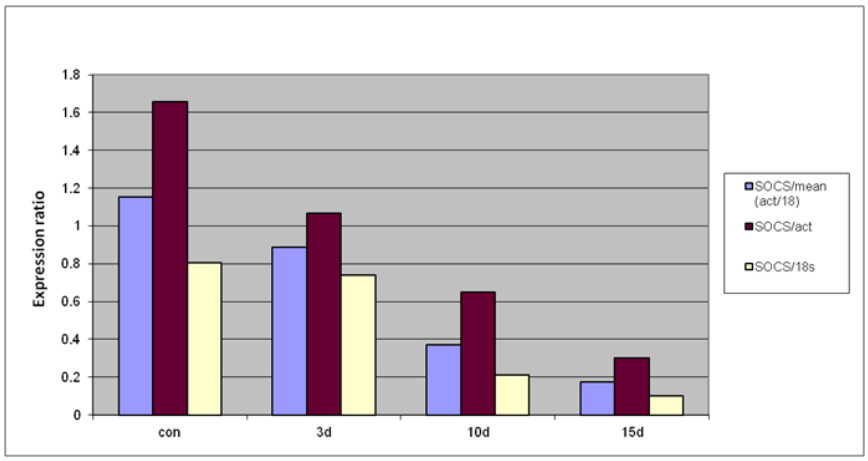
- Expression of sabinene synthase (SoSS) in detached middle-aged sage leaves



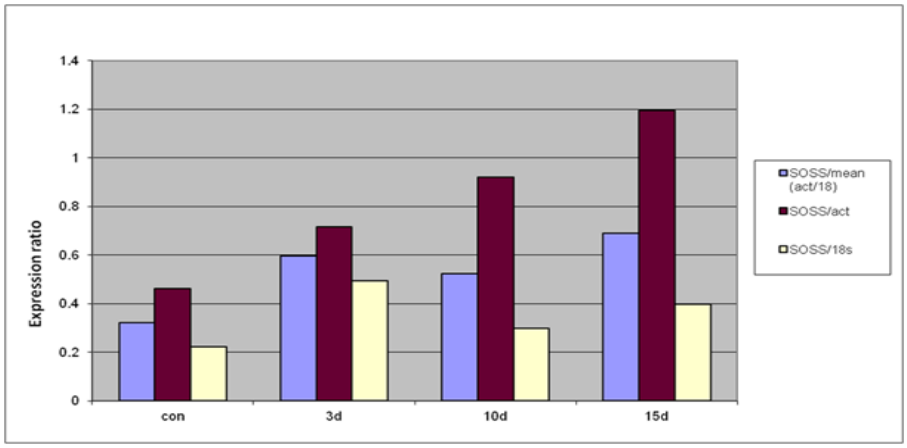
- Expression of bornyl diphosphate synthase (SoBS) in intact sage leaves (Incubation time by days)



- Expression of cineole synthase (SoCS) in intact sage leaves



- Expression of sabinene synthase (SoSS) in intact sage leaves



- **Figure: GC- chromatogram. The retention time was 3.32, 4.68, 5.6 and 6,48 for cineole, α- β-thujone, camphor and tetradecan, respectively.**